Regulation of Inflammation Genetic Control of the Transcription of Spätzle in Drosophila Hemocytes

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Regulation of Inflammation

Genetic Control of the Transcription of *Spätzle* in *Drosophila* Hemocytes

By

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ABSTRACT

The *Drosophila* cytokine *Spätzle* (*Spz*) triggers the Toll signalling cascade in flies upon infection with Gram-positive bacteria and fungi. It is an analogue to the mammalian IL-1 and is produced by the fat body as a precursor protein (Spz-precursor). Active Spz is produced from its precursor by a tightly regulated cascade of serine proteases. However, the transcriptional regulation of *Spz* in *Drosophila* in response to bacterial or fungal infection is still poorly deciphered. Therefore, the aim of the project was to interrogate the regulation of *Spz* transcription, with specific interest in hemocytes, which are equivalent to mammalian monocytes/macrophages. For this purpose, we have generated and characterised a transgenic Spz-neGFP reporter that allows the visualisation of *Spz* transcription in individual cells and tissues during different stages of development. We have identified populations of cells that transcribe the *Spz* gene in the steady state throughout development. We have infected the transgenic reporter flies with the fungus *Candida albicans* and examined the transcription of *Spz* after immune challenge. In future, these strains and results will help develop an *in vivo* genome wide assay, employing *Drosophila* RNAi libraries, in order to gain more knowledge about the transcriptional regulation of *Spz* and to identify novel regulatory genes and pathways for *Spz* transcription.

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DECLARATION

All the work described in this thesis was conducted by the author in the laboratory of Professor Frederic Geissmann and Dr. Marc Dionne from October 2008 to July 2012 in the Centre for Molecular and Cellular Biology of Inflammation (CMCBI), School of Medicine, King's College London. The contents are the original work of the author except when indicated. The contents have not been previously submitted for any degree or qualification at another university or institution.

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ABBREVIATIONS

AMPs	Antimicrobial peptides
Antp	*Antennapeadia*
BSA	Bovine serum albumin
C. albicans/Candida	*Candida albicans*
CD	Cluster of differentiation
Col	Collier
CrPV	Cricket paralysis virus
Crq	Croquemort
DAP-PGN	Diaminopimelic acid type peptidoglycan
daw	Dawdle
Dif	Dorsal-related immune factor
DNA	Deoxyribonucleic acid
dpp	Decapetaplegic
DsRed	*Discosoma* red fluorescent protein
Drosophila	*Drosophila melanogaster*
E. coli	*Escherichia coli*
Ecc15	*Erwinia carotovora carotovora*
eGFP	Enhanced green fluorescent protein
FLP	Flippase recombination enzyme
FOG	Friend of GATA
FRT	*Flippase recognition target*
Gcm	Glial cell missing
GFP	Green fluorescent protein
GNBP	Gram-negative binding protein
Hml	Hemolectin
Hth	Homothorax
HR	Homology region
ICE	Interleukin-1 converting enzyme
IκB	Inhibitor of NF-κB
IL	Interleukin

IL-1R	Interleukin-1 receptor
IRES	Internal ribosome entry site
Imd	Immune Deficiency
IRAK	IL-1R associated kinase
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
KI	Knock in
KO	Knock out
LB	Lysogeny broth
Lz	Lozenge
Lys-PGN	Lysine-type peptidoglycan
mCherry	Monomeric Cherry, a red fluorophore
M. luteus	*Micrococcus luteus*
mRNA	Messenger RNA
MyD88	Myeloid differentiation primary response gene (88)
neGFP	Nuclear enhanced green fluorescent protein
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NGS	Normal goat serum
nls	Nuclear localisation signal
OD	Optical density
OR	Oregon-R
pBS	pBluescriptKS+
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PGN	Peptidoglycan
PGRP	Peptidoglycan recognition protein
Pirk	Poor Imd response upon knock-in
PRR	Pattern recognition receptor
PSC	Posterior signalling centre
Psh	Persphone

Pvf	PDGF/VEGF factor
Pvr	PDGF- and VEGF-receptor related
qPCR	Quantitative polymerase chain reaction
RedStinger	A vector that drives expression of nuclear DsRed
RFP	Red fluorescent protein
RIP	Receptor interacting protein
RNA	Ribonucleic acid
RNAi	RNA interference
Rpl1	Ribosome protein L4
Ser	Serrate
Serpin	Serine protease inhibitor
Sn	Singed
SPE	Spätzle processing enzyme
Spz	Spätzle
Sp7	Serine protease 7
Srp	Serpent
STAT	Signal transducer and activator of transcription
S2	Schneider 2 cells, a Drosophila hemocyte cell line
TAB	TAK1 binding protein
TAK	TGF-β activated kinase
TIR	Toll/IL-1R
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TRAF	Tumour necrosis factor receptor-associated factor
UAS	Upstream activation sequence
Upd	Unpaired
Upd3	Unpaired 3
Ush	U-shaped
VML	Vitelline membrane-like
Wg	Wingless
YPD	Yeast extract peptone dextrose medium

- Chapter 1 -
Introduction

1.1 Prologue

The immune system has evolved to protect the host from invading pathogens. In vertebrates, the innate immune system is the first arm of immunity recognises and provides non-specific but immediate defence against pathogens in a generic way. Besides, innate immunity is also required for the activation of a more specific form of immune system known as the adaptive immune response through the antigen presentation process. Lack of innate immunity causes severer health problems as majority of infections are controlled by the innate immune response before the activation of adaptive immunity. Cytokines – a chemical mediator that are produced mainly by immune cells as a result of innate immunity activation upon infection and/or injury – orchestrate the inflammatory response. However, the mechanism that regulates cytokine transcription in immune cells, such as monocytes/macrophages, during infection and inflammation is still poorly deciphered. Therefore, gaining a deeper insight into cytokine transcription can help to obtain a better understanding on the pathology of inflammatory diseases such as atherosclerosis and rheumatoid arthritis, in addition to help defining future therapeutic strategy.

The fruit fly *Drosophila melanogaster* is one of the most extensively studied and common experimental animal models in the field of biological science. It has been broadly used to understand the complex molecular and cellular mechanisms of many physiological processes such as the innate immune system. Similar to most invertebrates, *Drosophila* is highly resistant to infection albeit in the absence of any known form of acquired immune system. Without the influence of adaptive

immunity, *Drosophila* is an excellent model to unveil the complex regulation of innate immune response. The innate immune responses of *Drosophila* share many similarities to the mammalian innate system, particularly the mammalian Toll-like receptor (TLR) signalling cascade that highly resembles the Toll pathway in fly. The Toll pathway is one of the most potent and influential signalling cascades in *Drosophila*. Toll and its ligand Spätzle was first discovered as a developmental pathway that controls the dorsal-ventral polarization during early embryogenesis. Later, the significance of Toll and Spätzle in protecting the fly against fungal and Gram-positive bacteria was reported. Upon detection of infection, circulating Spätzle is activated through proteolytic cleavages and triggers the downstream Toll pathway by binding to its receptor Toll located on the surface of the fat body. Fat body has long been identified as the primary tissues where immune responses take place and subsequently lead to synthesis of antimicrobial peptides. Only until recently, evidences suggested that the phagocytic hemocytes are capable of secreting cytokines to communicate with fat body and hence initiate the systemic immune responses upon detection of infection and inflammation. To date, three *Drosophila* cytokines have been identified – Spätzle (Spz), an IL-1 analogue; Unpaired3 (Upd3), a potential IL-6 analogue that stimulates the downstream JAK/STAT signalling pathway; and Eiger (Egr), an analogue to the mammalian Tumour necrosis factor (TNF) and ligand to the fly JNK pathway. Except Spz, which relationship with Toll has been extensively studied due to its vital role in embryo development and anti-fungal capability, knowledge on Upd3 and Egr are very limited. Previous studies have demonstrated secretion of Spz and Upd3 by hemocytes upon fungal infection and wounding, respectively. But no direct evidence is yet available to show production of Egr by hemocytes.

The machinery between hemocytes and fat body is highly similar to the mammalian system in which innate immune cells, such as monocytes/macrophages, transcribe and produce cytokines to activate systemic immunity following recognition of pathogenic components externally and/or internally. However, the mechanisms underlying in such process in hemocytes and monocytes/macrophages are poorly understood. Therefore, this project is aiming to obtain better understanding in cytokine transcription upon immune challenge and further dissect the genetic regulation of cytokines in hemocytes during infection and inflammatory events.

1.2 The Immune System of Drosophila

Drosophila has been used as a model to study innate immunity. *Drosophila* has an 'innate immune system' that resembles the vertebrate innate immune system both at the cellular and molecular levels. The fly immune system responds to tissue damage and infection through two different mechanisms – the humoral immune response and the cellular immune response.

1.2.1 The Humoral Immune Response

The humoral immune response of *Drosophila* involves melanisation and activation of two distinct signalling pathways in the fat body that eventually lead to the production of antimicrobial peptides (AMPs). These two pathways are the Toll, which is activated by Gram-positive bacteria and fungus infection, and Immune Deficiency (Imd) pathways that protect the fly against infection by Gram-negative bacteria. These pathways can be activated in two ways: by a soluble or membrane-bound peptidoglycan recognition receptor to recognise the microbes; or a signal, such as the fly cytokine Spätzle, released by hemocytes in response to immune challenges (Brennan and Anderson, 2004; Hoffmann, 2003; Hoffmann and Reichhart, 2002; Kimbrell and Beutler, 2001; Tanji et al., 2007; Tanji and Ip, 2005).

1.2.1.1 Spätzle and the Toll Pathway

Besides its important role in defining the dorsal-ventral polarity during embryogenesis (Anderson et al., 1985; Govind and Steward, 1991; Hashimoto et al., 1991), post-development the Toll pathway protects the fly against infection with Gram-positive bacteria, yeast and fungi through activation of the downstream

MyD88/NF-κB pathway that eventually leads to the production of antimicrobial peptides (AMPs) (Lemaitre, 2004; Valanne et al., 2011). The participation of Toll in fly innate immune responses was initially speculated due to the discovery of κB-like binding motifs upstream of the AMPs Cecropin and Diptericin that were inducible as a consequence of bacterial infection (Engstrom et al., 1993; Kappler et al., 1993; Reichhart et al., 1992). However, the contribution of Spz and Toll to the immune response was not confirmed until 1996 when Bruno Lemaitre and Jules Hoffmann characterised it completely (Lemaitre et al., 1996). By infecting adult *Drosophila* of a Toll mutant background with fungi *A. fumigatus*, they reported that Toll knock down flies were unable to produce Drosomycin, one of the seven AMPs, and had a reduced survival rate.

Toll and its downstream signalling cascade have been shown to be evolutionarily conserved to the mammalian Toll-like receptor (TLR) signalling pathways. To date, nine Toll proteins have been identified in *Drosophila* and a structural similarity at the cytoplasmic domain is shared between Toll, also known as Toll 1, and human interleukin-1 (IL-1) receptor (IL-1R) (Gay and Keith, 1991; Schneider et al., 1991). All of the Toll protein family members in *Drosophila* share conserved leucine-rich repeats and cysteine-rich motifs in the extracellular domain and can be divided into different sub-groups according to their structural similarity at the ectodomain (Tauszig et al., 2000). For instance, Toll 5, also named Tehao, shares 60% similarity with Toll, making it the closest family member to Toll (Tauszig et al., 2000) and the downstream signalling events appear to be shared between Toll and Toll 5 (Luo et al., 2001). Most of the Tolls are expressed mainly during embryonic stages and thus possess a putative developmental role. Among these nine Toll

proteins, Toll is the major participant in response to bacterial and fungal infection and responsible for Drosomycin, an antimicrobial peptide, synthesis; whereas the detailed functions of other Tolls are yet to be defined (Imler and Hoffmann, 2001).

Activation of the Toll pathway is triggered by the binding of its cytokine ligand – Spätzle. Spz belongs to the cysteine-knot family and is most similar to the nerve growth factor coagulogen (Mizuguchi et al., 1998). The inactive Spz composes of a 25kDa pro-domain, which is required for protein secretion, and a 14kDa C-terminal domain for receptor association (DeLotto and DeLotto, 1998; Weber et al., 2007). It circulates in the hemolymph as a dimeric pro-protein with a disulphide bond linking the two Spz pro-proteins together at the C-terminal domain (Weber et al., 2003), and is activated through serine protease cleavage (Schneider et al., 1994). Proteolytic cleavage by serine proteases cleaves the pro-Spz between R143 and V144 at the C-terminal, generating a C-terminal fragment of 106 amino acids (C-106). This also induces conformational change at the C-terminal domain revealing a masked surface in the C-106 centred on a unique tryptophan residue that is essential for binding the Toll protein and establishing the downstream signalling process (Arnot et al., 2010; DeLotto and DeLotto, 1998). Despite its role in assisting pro-Spz secretion into the hemolymph post translational modifications in the endoplasmic reticulum, the pro-domain of Spz is also required to establish a stable and tight binding between the active C-106 domain and the Toll receptor (Weber et al., 2007).

In response to fungal and bacterial infection, Spz is processed by proteolytic cleavage performed by Spätzle Processing Enzyme (SPE) (Jang et al., 2006). Upon

recognition of Gram-positive bacterial and fungal derivatives by various soluble pattern recognition receptors in the hemolymph, a cascade of serine protease cleavages is initiated to activate Spz. Two groups of pattern recognition molecules have been described in fruit fly, the Peptidoglycan Recognition Protein (PGRP) and the Gram-negative Binding Protein (GNBP), that work alone or together to recognise non-self agents and elicit the downstream Toll and Imd pathways (Aggrawal and Silverman, 2007; Charroux et al., 2009; Kurata, 2004; Leulier et al., 2003).

Lysine-type Peptidoglycan (Lys-PGN) is an essential cell wall component of Gram-positive bacteria and is recognised by PGRP-SA, PGRP-SD, and GNBP 1. PGRP-SA is the first peptide recognition receptor identified for sensing Gram-positive bacteria in *Drosophila*. Inactive PGRP-SA due to mutation in the *Semmelweis* gene impairs the Toll pathway in responding to Gram-positive bacterial, but not fungal, infection (Michel et al., 2001). GNBP 1, is also required to detect Lys-PGN because a disrupted GNBP 1 gene results in a compromised immune response against infection with Gram-positive bacteria (Gobert et al., 2003; Pili-Floury et al., 2004). GNBP 1 interacts with PGRP-SA during the bacterial recognition process but its precise function is yet to be determined. One of the proposed functions is to hydrolyse the Lys-PGN to muropeptides so that PGRP-SA can bind to the processed and purified fragments with higher affinity (Filipe et al., 2005; Wang et al., 2006). In addition, an enhanced interaction was observed between PGRP-SA and GNBP 1 in the presence of Lys-PGN. This is potentially due to the generation of a new molecular surface caused by the binding of PGRP-SA and the Lys-PGN, hence allowing binding of GNBP 1 to the complex (Chang et al., 2004; Wang et al., 2006).

However, as the enzymatic ability of GNBP 1 is only restricted to certain strains of Gram-positive bacteria, for example *Micrococcus luteus* (*M. luteus*), it has recently been suggested that GNBP 1 acts as a bridging protein between PGRP-SA and the novel serine protease ModSP, which acts upstream of Spz production by SPE (Buchon et al., 2009). In parallel to the PGRP-SA/GNBP 1 complex, PRGP-SD also detects Lys-PGN alone or in partial redundancy to PGRP-SA and GNBP 1. In the absence of PGRP-SA and GNBP 1, activation of the Toll pathway was reported after challenge with *Staphylococcus saprophyticus* indicating the existence of a novel sensor – PGRP-SD – that recognises bacterial components in a PGRP-SA/GNBP 1 independent manner. Moreover, the PGRP-SD loss of function mutant has shown to be unable to modulate Drosomycin production via the Toll pathway in response to *S. pyogenes* and *S. aureus* infection (Bischoff et al., 2004). Besides, PGRP-SD is also able to enhance the Toll signalling cascade by stabilizing the interaction between PRGP-SA and GNBP 1 and/or forming a ternary complex with PRGP-SA and GNBP 1 (Wang et al., 2008). The role of PGRP-SD in bacterial recognition and regulation of the Toll pathway is still very ambiguous since it can potentially function as a receptor for Gram-negative bacteria. The crystal structure of the PGRP-SD protein suggested a preference in binding Diaminopomelic acid-type peptidoglycan (DAP-PGN), a major structural protein found in the cell wall of Gram-negative bacteria, rather than Lys-PGN *in vitro* (Leone et al., 2008). This might suggest a cross talk between the Toll pathway and the Imd pathway at the level of bacterial recognition receptor and more investigation in the PGRP-SD protein is therefore needed.

Two independent mechanisms have been described for fungal detection in *Drosophila*. Both mechanisms recognise fungi using different receptors but activate

Spz through SPE-mediated cleavage (Gottar et al., 2006). GNBP 3 detects fungal infection in fly via binding to fungal polysaccharide β-(1,3)-glucans (Kim et al., 2000). Flies that lack GNBP 3 expression are intolerant to *Candida albicans* infection but show no incapability in fighting Gram-positive and Gram-negative bacteria (Gottar et al., 2006). Recognition of β-(1,3)-glucans by the GNBP 3 protein triggers activation of the serine protease ModSP and hence leads to the onset of the Toll signalling cascade (Buchon et al., 2009). In addition to the GNBP 3 protein, *Drosophila* is also protected from fungal infection via recognition of the fungal virulence factor PR 1 protease by Persephone, a serine protease that directly regulates SPE cleavage in a ModSP independent manner (Gottar et al., 2006; Levashina et al., 1999; Ligoxygakis et al., 2002). PR 1 is a fungal protease secreted for cuticle penetration during spore deposition on the fly surface (Bagga et al., 2004; Wang et al., 2005). Besides fungal protease, Persephone is also able to sense proteases synthesised by Gram-positive bacteria (El Chamy et al., 2008). The proteolytic activity of Persephone is regulated by the serine protease inhibitor (serpin) Necrotic. In flies of necrotic mutant background, active Spz is constitutively expressed leading to constant activation of Toll, indicating Necrotic is negatively regulating the Toll pathway (Levashina et al., 1999). In response to fungal and Gram-positive bacterial infection, the N-terminal domain of necrotic is cleaved resulting in the release of Persephone and thus initiates the downstream Toll signalling process (Pelte et al., 2006). However, the mechanism employed to induce this cleavage is yet to be determined. Taken together, the Persephone-mediated cascade works in parallel with the ModSP-dependent cascade to modulate Spz cleavage by SPE. This cascade is especially important in defeating pathogenic immune challenges as it not only recognises internal infectious agents,

but also detects external infectious agents, providing complete protection to *Drosophila* against fungal and Gram-positive bacteria infection.

Following recognition of Lys-PGN and β-(1,3)-glucans by PGRP-SA/GNBP 1/PGRP-SD and GNBP 3, respectively, a chain of protease cleavages is initiated in order to integrate signals received from the pattern recognition receptors for Toll activation. ModSP, a modular serine protease, is located at the top of this cascade and is responsible for receiving signals from the receptors (Buchon et al., 2009). The signal is then transduced downstream through four serine proteases Grass, Spirit/Spheroide/Sphinx 1/2 and finally, the CLIP domain of serine protease SPE (Kambris et al., 2006). SPE cleaves the inactive pro-Spz into active Spz for Toll binding during immune responses (Jang et al., 2006). During embryonic development and larval stages, SPE is mainly expressed by the fat body and is positively regulated by the Toll pathway since production of SPE post infection is disrupted in Toll mutant (Mulinari et al., 2006). Active Spz dimer binds to Toll at the N-terminus of the ectodomain and indirectly dimerises the receptors between the N-terminus of the solenoid and the C-terminal juxtamembrane sequence by inducing conformational changes (Gangloff et al., 2008; Weber et al., 2005). The intracellular Toll signalling cascade is conserved between dorsal-ventral polarization and innate immune responses. MyD88 and Tube are required to interact with Toll and act as linking proteins for the protein kinase Pelle (Horng and Medzhitov, 2001; Medzhitov et al., 1998; Tauszig-Delamasure et al., 2002). Degradation of the IκB inhibitor Cactus releases the NF-κB transcription factors, Dorsal and Dif (Dorsal-related Immune Factor), in which Dif is the major trigger

for the transcription of immune response genes like Drosomycin by binding to the κB-like sequence motifs (Ip et al., 1993; Rutschmann et al., 2000a) (Fig. 1-1).

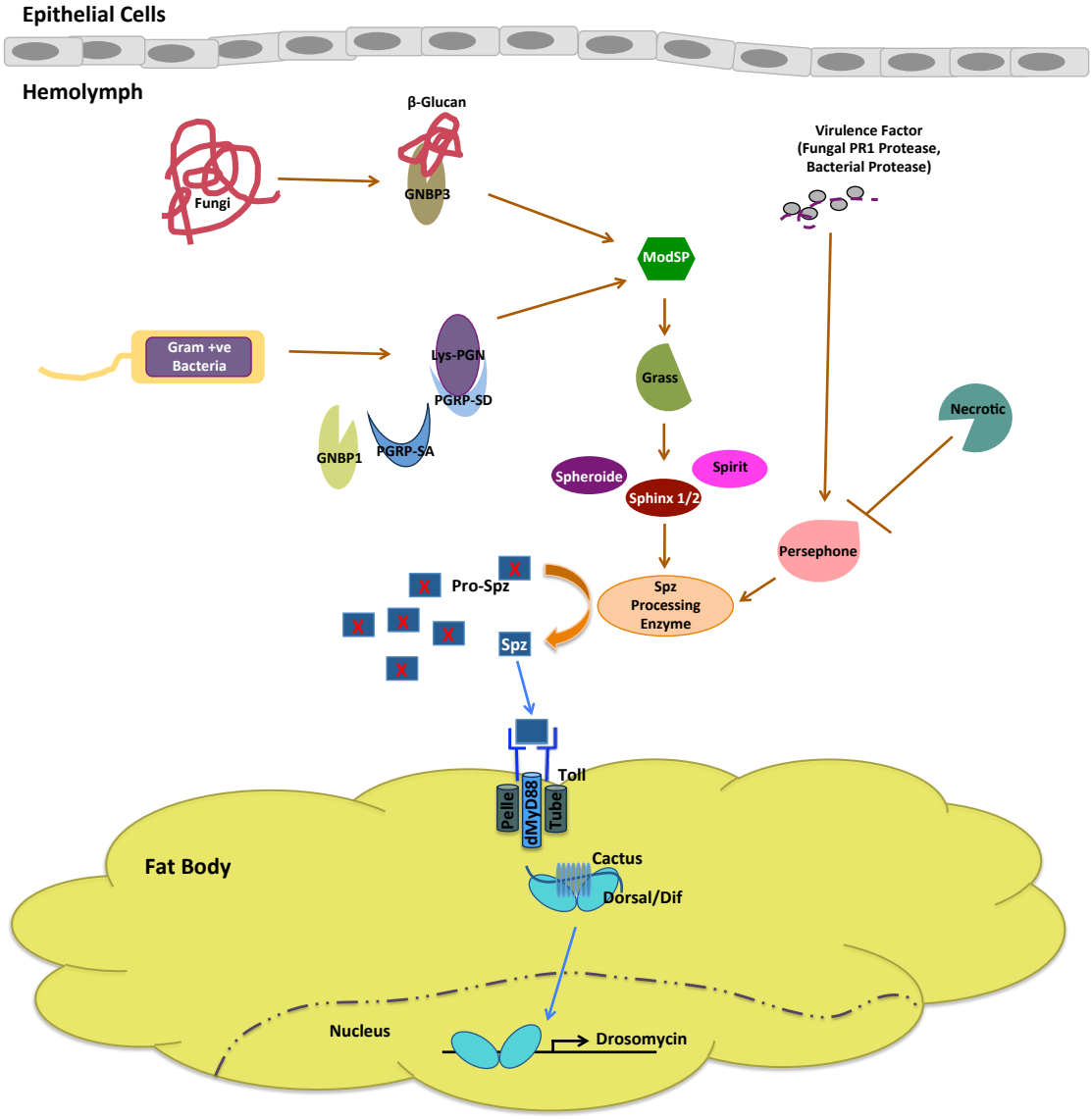


Figure 1-1: ***Activation of the Toll pathway during Gram-positive bacteria and fungal infection.*** Detection of intracellular fungal and bacterial components by specific circulating pattern recognition receptors in the hemolymph activates a chain of serine proteases cascade that eventually leads to the cleavage of Spz processing enzyme (SPE). An additional mechanism is used to recognise protease synthesised by both fungus and bacteria and is able to modulate SPE activity directly in a ModSP independent manner. Activated SPE cleaves the inactive pro-Spz at the C-terminal domain and hence turns it into the active form. Proteolytically processed Spz binds to the transmembrane Toll receptor on the fat body cells and triggers the downstream intrinsic signalling pathway that consequently leads to the production of antimicrobial peptide Drosomycin.

1.2.1.2 Comparison of the Drosophila Toll Pathway to the Mammalian IL-1R Signalling Cascade

The Toll pathway in *Drosophila* and the mammalian IL-1 (interleukin-1) pathway represent an evolutionary conserved intercellular signalling cascade that is used in host immune responses. Activation of both pathways occurs through the transmembrane receptor – Toll in *Drosophila* and IL-1R (interleukin-1 receptor) in mammals – following detection of microbial components and leading to translocation of the transcription factor NF-κB into the nucleus. The cytoplasmic domain of Toll and IL-1R are highly conserved with 45% of the amino acid sequence being identical or conservatively substituted (Gay and Keith, 1991), and hence they are termed as the Toll/IL-1R (TIR) domain (O'Neill and Greene, 1998). IL-1R belongs to the mammalian Toll-like receptor (TLR) superfamily (O'Neill, 2008). The cytokine IL-1 is the known ligand for IL-1R and production of the cytokine by various immune cells like monocytes can be modulated by multiple infectious agents and during inflammatory responses. Due to the similarity between the Toll and IL-1R pathway, the pro-inflammatory IL-1 is proposed to be the analogue of Spz. Two types of IL-1 have been identified, IL-1α and IL-1β, and both bind to IL-1R (Auron et al., 1984; Lomedico et al., 1984; March et al., 1985). An IL-1 inhibitor known as IL-1 receptor antagonist (IL-1Ra) has been characterised and the three proteins, IL-1α, IL-1β and IL-1Ra complete for IL-1R occupancy upon infection and inflammation (Carter et al., 1990; Eisenberg et al., 1990). Both IL-1α and IL-1β are synthesised as a precursor protein (proIL-1α and proIL-1β) of 31kDa that is converted into an active form of 17kDa through enzymatic cleavage by the caspase interleukin-1 converting enzyme (ICE) upon detection of microbial components (Dinarello, 1994; Nett et al., 1992; Thornberry

et al., 1992). Binding of active IL-1 to IL-1R engages downstream signalling transduction. Similar to the *Drosophila* Toll pathway, the downstream intracellular signalling cascade begins with recruitment of the adaptor protein MyD88 to the cytosolic TIR domain (Wesche et al., 1997). MyD88 links the TIR domain of the IL1-R to the protein kinase IRAK 1, homologous to Pelle in flies, and the death domain of IRAK4 (Cao et al., 1996; Wesche et al., 1997). The IRAK complex then detaches from MyD88 and interacts with the E3 ubiquitin ligase tumour necrosis factor receptor-associated factor 6 (TRAF6) (Deng et al., 2000). This leads to auto-ubiquitination causing recruitment of a kinase complex composed of TGF-β-activated kinase 1 (TAK1) and TAK1 binding protein (TAB) (Deng et al., 2000; Ninomiya-Tsuji et al., 1999). Subsequently, TAK1 triggers degradation of IκB, a NF-κB-inhibitory protein, through phosphorylation on two key serine residues Ser^{32} and Ser^{36} (Verma et al., 1995). Degradation of IκB results in the release of NF-κB and allows nuclear translocation of the transcription factor, which consequently regulates expression of target genes (Dunne and O'Neill, 2003; O'Neill, 2008; O'Neill and Greene, 1998) (Fig. 1-2).

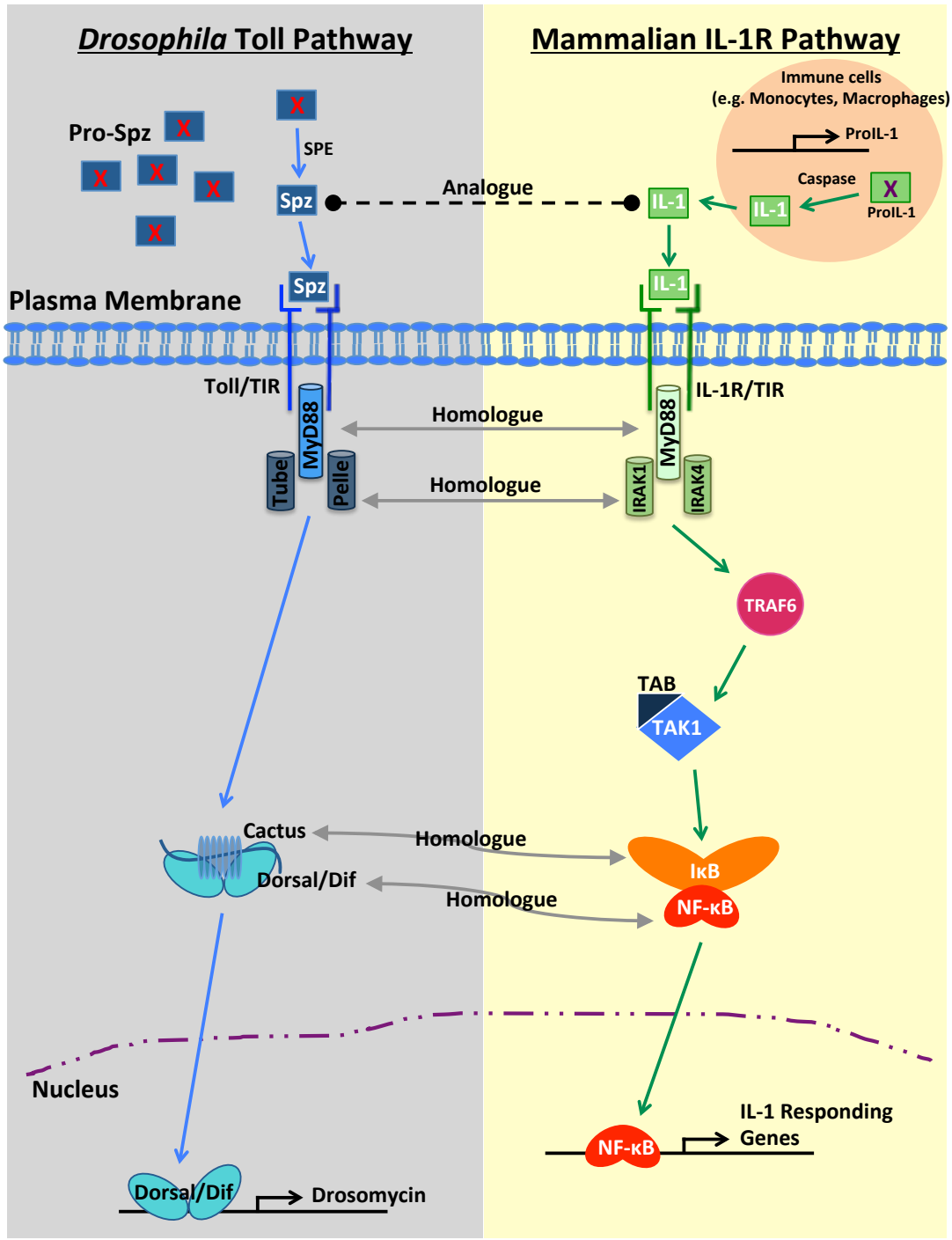


Figure 1-2: ***Comparison of the Drosophila Toll pathway to the mammalian IL-1R pathway.*** The cytoplasmic signal transduction of the *Drosophila* Toll pathway resembles the mammalian IL-1R pathway. Binding of active ligand to the receptor Toll and IL-1R by Spz and IL-1, respectively, mediates recruitment of the MyD88/IRAK complex to the cytosolic TIR domain. In *Drosophila*, interaction of the IRAK kinase Pelle to MyD88 triggers degradation of the NF-κB inhibitor Cactus, resulting in release of the transcription factors Dorsal and Dif and nucleus

translocation for Drosomycin expression. In mammal, the MyD88/IRAK1/IRAK4 complex acts a platform for the self-ubiquination of TRAF6 to allow recruitment of the TAK1 and TAB complex, which subsequently phosphorylates IκB to release NF-κB for transcription of IL-1 responding genes.

1.2.1.3 The Imd Pathway

The *Drosophila* Imd pathway orchestrates the immune response against Gram-negative bacteria infection (Kaneko and Silverman, 2005). The Imd pathway was first identified and described by Lemaitre et al. in 1995 when flies of a recessive mutation in the *Imd* gene background became susceptible to bacterial infection and showed impaired AMP expression (Corbo and Levine, 1996; Lemaitre et al., 1995). The Imd pathway employs the pattern recognition receptor PGRP-LC to detect bacterial cell wall components upon infection with Gram-negative bacteria (Choe et al., 2002; Gottar et al., 2002; Leulier et al., 2003; Ramet et al., 2002). Lack of functional PGRP-LC results in reduced survival after exposure to Gram-negative bacteria and decreased expression of AMPs, but no difference in susceptibility or AMP level when infected with Gram-positive bacteria or fungus (Choe et al., 2002; Gottar et al., 2002; Ramet et al., 2002).

PGRP-LC is a transmembrane protein able to recognise specifically Diaminopimelic acid type peptidoglycan (DAP-PGN), the most common form of PGN found in the cell wall of both Gram- positive and Gram-negative bacteria. Three isoforms of PGRP-LC, PGRP-LCa, PGRP-LCx and PGRP-LCy, have been reported in which each PGRP-LC shares identical sequence at their intracellular and transmembrane domain but varies at the PGRP motif in the extracellular domain (Werner et al., 2003). These isoforms are capable of forming homodimers or heterodimers during the recognition process with different bacterial cell wall components and thus provide a greater range of recognition ability through various combinations among these receptors (Kaneko et al., 2004; Kaneko et al., 2005). These data also suggest that a heterodimer of PGRP-LCa and –LCx is required to detect monomeric DAP-

PGN while a homodimer of PGRP-LCx will recognise and bind to polymeric DAP-PGN (Kaneko et al., 2004; Kaneko et al., 2005). Besides PGRP-LC, PGRP-LE has been shown to function synergistically together with PGRP-LC and is required for the activation of infection-dependent prophenoloxidase (proPO) cascade in flies (Takehana et al., 2004). Two distinct functions of PGRP-LE have been described according to their structure (Kaneko et al., 2006). PGRP-LE containing only the PGRP domain functions as an extracellular receptor. Together with PGRP-LC, it is able to recognise extracellular monomeric PGN and thus enhances PGRP-LC-mediated PGN recognition on the cell surface, similar to the mammalian CD14 molecule. Full length PGRP-LE, on the other hand, is located inside the cell acting as an intracellular receptor for monomeric PGN (Kaneko et al., 2006).

Binding of DAP-PGN by PGRP-LC triggers the intrinsic signalling cascade through the Imd protein, a 30 kDa death domain protein that is most similar to the mammalian Receptor Interacting Protein (RIP) (Georgel et al., 2001). The protein is located at the most upstream position of the Imd pathway and is directly associated with PGRP-LC at its cytoplasmic domain (Choe et al., 2005). Downstream of the Imd protein sits a caspase named Dredd, a Drosophila homologue of the mammalian caspase 8, that is linked to Imd by a death adaptor protein FADD, which is most similar to the mammalian Fas-associated death domain-containing protein (Leulier et al., 2000; Leulier et al., 2002; Naitza et al., 2002).

Relish is the third known NF- κB described in flies among with Dorsal and Dif. It is a bipartite protein in which the N-terminal domain resembles the mammalian Rel

domain, whereas the C-terminal domain is composed of ankyrin repeats and PEST-like sequence (Dushay et al., 1996). Unlike Dorsal and Dif, which are held in the cytoplasm by the IκB homologue Cactus in unchallenged condition, Relish is held in the cytoplasm by its own IκB-like domain in the C-terminus (Dushay et al., 1996). Hence, translocation of Relish into the nucleus requires endoproteolytic cleavage. Dredd associates with Relish and cleaves the protein at its C-terminal upon DAP-PGN stimulation (Erturk-Hasdemir et al., 2009; Stoven et al., 2000; Stoven et al., 2003). The IKK complex, which is located downstream of the Dredd/FADD complex and TAK1, mediates Relish protolysis. In this case, following detection of DAP-PGN by PRGP-LC, the caspase activity of Dredd is required to cleave the Imd protein at the caspase recognition motif $_{27}$LEKD/A$_{31}$ exposing a highly conserved inhibitor of apoptosis (IAP)-binding motif (IBM) on the neo-N terminus at A31 (Paquette et al., 2010). Cleaved Imd binds to Iap2 via the BIR domains in the N-terminal of Iap2 and rapidly becomes K63-polyubiquitinated by associating with the E2-ubiquitin-conjugating enzymes complex containing Ubc13, Uev1a and Effete (Gesellchen et al., 2005; Kleino et al., 2005; Leulier et al., 2006; Zhou et al., 2005). Acting downstream of the Imd/Iap3/K63 polyubiquitin complex, the *Drosophila* homologue of MAPKKK kinase, TAK1, is interacting with the transforming growth factor-activated kinase 1-binding protein (TAB) (Kleino et al., 2005; Vidal et al., 2001). The Imd pathway branches downstream of TAK1. One of the branches activates the *Drosophila* JNK leading to transcription of the AP1 genes (Park et al., 2004; Silverman et al., 2003). The other branch involves participation of the ~400kDa IKK complex that is necessary for the proteolysis and translocation of Relish downstream. The complex contains two subunits, IKKβ that is also known as ird5, and IKKγ, which is also termed kenny (Lu et al., 2001;

Rutschmann et al., 2000b). Activation of the complex promotes direct phosphorylation on Relish on serines 528 and 529 in the C terminus that is crucial for the proper transcription activation of Relish target genes through recruitment of RNA polymerase II to the promoters of AMPs genes (Erturk-Hasdemir et al., 2009). However, the phosphorylation process does not trigger cleavage of Relish or translocation of the protein into the nucleus but is dependent for this on Dredd-mediated cleavage (Erturk-Hasdemir et al., 2009). Phosphorylated and cleaved Relish is able to enter the nucleus to elicit transcription of the AMP genes, for example, Diptericin (Wu and Anderson, 1998). (Fig. 1-3)

The Imd pathway is tightly regulated by various regulators in both a positive and a negative manner at different levels in the signalling cascade. The first two regulators, named Sickie and Dnr-1, were identified in a genome-wide RNAi screen conducted in S2 cells. Sickie is a positive regulator that regulates Relish activation by Dredd, whereas Dnr-1 forms a negative feedback loop by inhibiting the caspase activity of Dredd while Dnr-1 level is downregulated by Dredd (Foley and O'Farrell, 2004). Caspar downregulates the Imd pathway through preventing Relish from entering the nucleus and hence blocking AMP gene transcription (Kim et al., 2006). Expression of Pirk (poor Imd response upon knock-in) is induced in a Relish-dependent manner during Gram-negative bacteria infection and it impairs the pathway by interacting with Imd protein and the cytoplasmic domain of PGRP-LC and thus regulating the signal transduction process to prevent over induction (Kleino et al., 2008). It also regulates the Imd pathway by interacting with the PGRP-LC and PGRP-LE heterodimer (Aggarwal et al., 2008). Finally, Pirk is required to deplete PGRP-LC expression on the cell membrane by direct coupling

with the recognition protein. These activities of Pirk are required in order to discriminate pathogenic bacteria from commensal bacteria, and therefore establishing immune tolerance to normal gut flora (Lhocine et al., 2008).

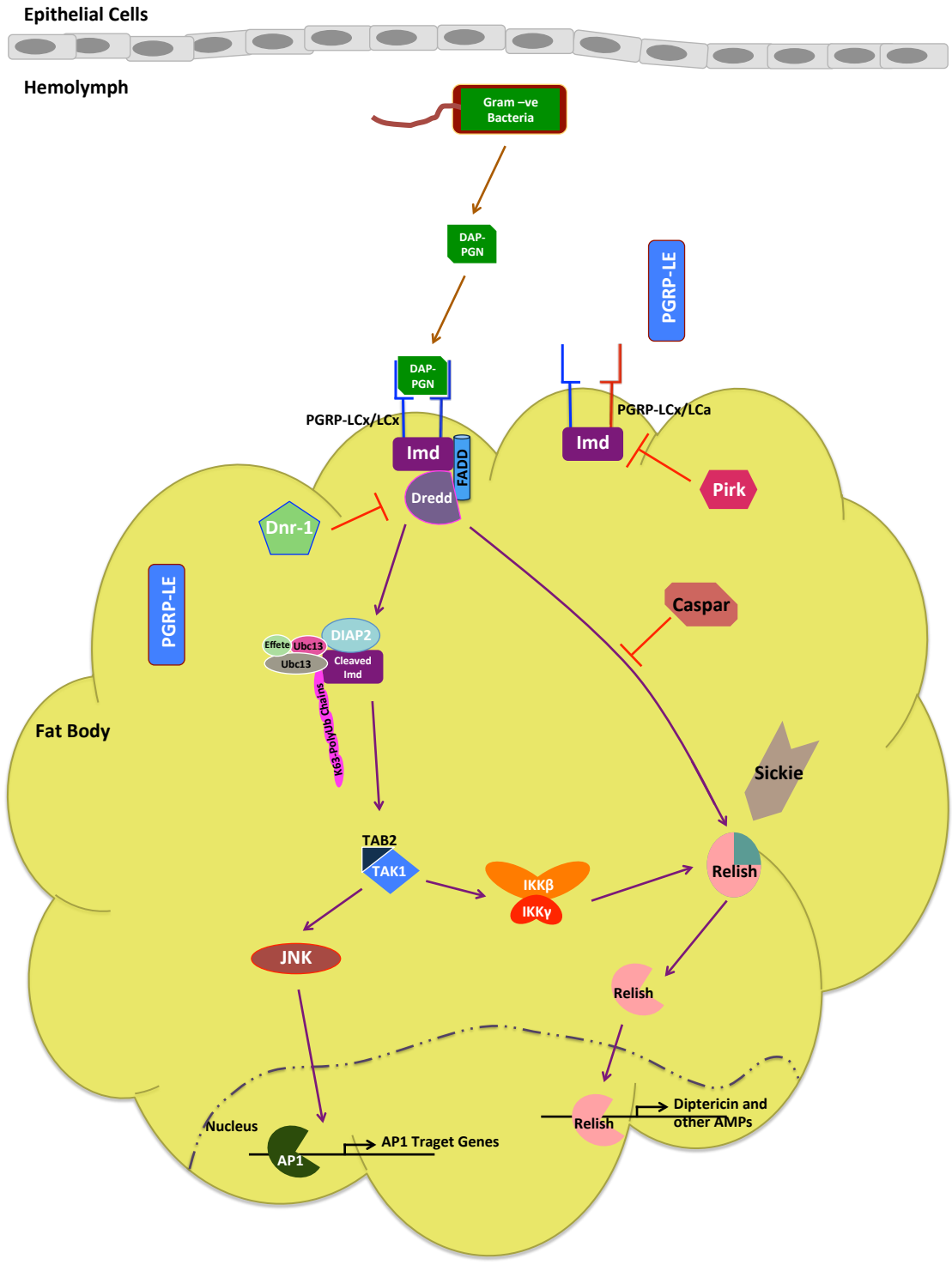


Figure 1-3: ***Activation of the Drosophila Imd pathway in response to infection with Gram –ve bacteria.*** Homodimer of PGRP-LCx or heterodimer of PGRC-LCx/LCa on the cell surface of the fat body tissue is able to recognise polymeric DAP-PGN and monomeric DAP-PGN, respectively. Binding of DAP-PGN to PGRP-LC triggers cleavage of Relish via two distinct pathways – direct proteolysis mediated by Dredd or cleavage mediated by the IKK complex. The activity of the IKK

complex is regulated by the upstream DIAP2-K63 polyubiquitin complex through the *Drosophila* MAPKKK kinase TAK1. In addition, TAK1 also modulate the infection-dependent JNK pathway for the transcription activation of AP1 target genes. Endoproteolytic processed Relish can now enter the nucleus and elicit antimicrobial peptide gene transcription.

1.2.2 The Cellular Immune Response

The cellular immune response of *Drosophila* involves the activities of fly blood cells – hemocytes. Two waves of haematopoiesis occur during early embryonic development and at larval stages giving rise to three different populations of hemocytes: plasmatocytes, crystal cells, and lamellocytes (Crozatier and Meister, 2007; Evans et al., 2003; Krzemien et al., 2010a; Meister and Lagueux, 2003; Wood and Jacinto, 2007).

1.2.2.1 Drosophila Haematopoiesis

1.2.2.1.1 The First Wave of Haematopoietic Process – The Embryonic Haematopoiesis

The first wave of blood cell development begins during early embryonic stages. It is originated at the procephalic mesoderm at the head before the blastoderm stage (Tepass et al., 1994). Mutation of either the *Bicaudal D* gene or both the *Snail* and *Twist* genes results in no hemocytes (Tepass et al., 1994). Differentiation of the mesoderm anlage into hemocyte precursors requires expression of Serpent (Srp), a GATA transcription factor, at the head area in the embryo (Lebestky et al., 2000; Rehorn et al., 1996). Maturation of prohemocytes into plasmatocytes and crystal cells relies on the expression of three conserved transcription factors, the RUNX transcription factor Lozenge (Lz), the FOG transcription factor U-shaped (Ush) and Glial cell missing (Gcm) 1 and 2 (Alfonso and Jones, 2002; Bataille et al., 2005; Lebestky et al., 2000). Embryonic hemocytes are first detected as early as embryonic stage 5, at which time all prohemocytes express both Srp and Gcm. Gcm expression in the anteriormost row of prohemocytes is downregulated followed by increased Lz expression in these prohemocytes by stage 7. Gcm expression in

hemocyte precursors promotes differentiation of prohemocyte into plasmatocytes, whereas Lz and Ush regulate crystal cells maturation (Bataille et al., 2005; Fossett et al., 2001). By the end of the embryonic haematopoietic process, a fixed number of hemocytes – 700 plasmatocytes and 36 crystal cells – are formed. The plasmatocytes subsequently migrate out of the head region to populate the embryo while all crystal cells remain localised close to the proventriculus (Bataille et al., 2005; Lebestky et al., 2000; Tepass et al., 1994). The migration of plasmatocytes follows four distinct routes and the process is directed by the Pvr receptor, a *Drosophila* homologue of PDGF/VEGF receptor tyrosine kinase that is expressed on all embryonic hemocytes (Cho et al., 2002; Heino et al., 2001; Tepass et al., 1994). Three Pvr ligands, Pvf 1-3, have been identified (Duchek et al., 2001). Activation of the Pvr pathway during embryonic haematopoiesis provides guidance to plasmatocytes for migrating out of the head towards the posterior end of the embryo while mutation of Pvr causes disruption in plasmatocyte migration. The three Pvfs are expressed along the migratory routes and expression of the ligands ectopically is able to reroute plasmatocytes to a new destination (Cho et al., 2002). In addition, Pvr regulates hemocyte survival during embryonic development. In the Pvr loss of function mutant, aggregation of plasmatocytes was observed, subsequently causing these cells to undergo apoptosis and eventually be phagocytosed by their peers (Bruckner et al., 2004).

1.2.2.1.2 The Second Wave of Haematopoietic Process – The Larval Haematopoiesis

The second phase of blood cell development in *Drosophila* takes place in a specific larval tissue - known as the lymph gland - that is of mesoderm origin. The *Drosophila* lymph gland is a population of cells that originate from the thoracic segments T1 to T3 of the lateral thoracic mesoderm that later attach themselves to the sides of the dorsal vessel after dorsal closure (Holz et al., 2003; Mandal et al., 2004). This cluster of embryonic lymph gland precursors are defined by their expression of the homeotic gene *Antennapeadia* (*Antp*), its downstream target Collier (Col), Odd-skipped that is expressed after Col, and Srp, whose expression is detected only after the formation of the primary lobes and is maintained throughout larval development (Crozatier et al., 2004; Jung et al., 2005; Lebestky et al., 2000; Mandal et al., 2007). In the late embryo, the lymph gland comprises of a single pair of lobes, namely the primary lobes, located on each side of the dorsal vessel. The Posterior Signalling Centre (PSC) is located on the posteriormost end of each of the primary lobes. Development of the PSC in larvae is dependent on *Antp* and Col expression. *Antp* and Col expression is initially expressed by all lymph gland precursors but towards to the end of lymph gland development is exclusive to a specific population of cells. Restricted expression of *Antp* and Col at cells located at the posterior end of the lymph gland specifies the anlage and subsequently determines PSC formation (Crozatier et al., 2004; Mandal et al., 2007). Antp expression is regulated by the homeodomain cofactor Homothorax (Hth) in a mutually exclusive manner and hence it is believed that Antp expression shapes the PSC, whereas Hth expression defines the rest of the lymph gland (Mandal et al., 2007).

In third instar larvae, the mature and functioning lymph gland comprises four paired compartments situated along the dorsal vessel: the primary lobes that can further be subdivided into the medullary zone and the cortical zone; the PSC that is located at the posterior end of the primary lobes; the secondary lobes; and the tertiary lobes. The medullary zone is sited at the inner core of the primary lobes containing a pool of prohemocytes that are awaiting for differentiation signals in order to develop into different populations of hemocytes, whereas the cortical zone that occupies the outskirts of the primary lobes acts as a reservoir for differentiating and differentiated hemocytes, including crystal cells and lamellocytes, before metamorphosis. Under infectious and inflammatory conditions, mature hemocytes will be released into the hemocoel from the cortical zone. The secondary and tertiary lobes mainly contain prohemocytes that will be moved up to the medullary zone when undergoing immune challenge (Jung et al., 2005). A recent study by Krzemien et al. has shown that a specific population of primordial hematopoietic cells originating from the embryonic lymph gland gives rise to the undifferentiated multi-potent hemocyte precursors in the medullary zone, and thus the fate of these prohemocytes to become plasmatocytes or crystal cells is committed during early lymph gland development (Krzemien et al., 2010b).

The PSC possesses a pivotal role during larval hematopoiesis and its function is very reminiscent of the mammalian stem cells niche. . The PSC is required for the maintenance of blood cell homeostasis in healthy larvae as well as for the control of lamellocyte differentiation from hematopoietic progenitors in response to parasitic infection with a wasp egg that is too large to be encapsulated by plasmatocytes (Crozatier et al., 2004; Duvic et al., 2002; Krzemien et al., 2007;

Mandal et al., 2007). . In larval lymph gland, the PSC is defined by the expression of Col and the Notch ligand Serrate (Ser) and the number of PSC cells is controlled and regulated by the Wingless (Wg) pathway (Sinenko et al., 2009) . Increased differentiation of plasmatocytes and crystal cells is observed in the Col mutant, eventually leading to premature loss of the medullary zone, while expression of Col in the Col-free medullary zone suppresses plasmatocyte and crystal cell differentiation. Thus, Col expression in the PCS is required for preventing prohemocyte differentiation. Col expression in the PCS is maintained by the Notch signalling pathway. Lack of Ser or Notch expression in the PCS results in the absence of Col in the third instar PCS (Krzemien et al., 2007). Activation of the Notch pathway by Ser promotes Lz expression and subsequently promotes crystal cells differentiation from prohemocytes (Duvic et al., 2002; Krzemien et al., 2010b).

In addition to the Notch pathway and Col, the JAK/STAT signalling pathway is also a key controller of the blood cell maturation process. Dome, the receptor for Unpaired (Upd), is expressed by prohemocytes in the medullary zone. Activation of the signalling pathway in prohemocytes by an as yet unknown signal from the PSC prevents premature differentiation of prohemocytes into plasmatocytes or crystal cells and hence ensuring the maintenance of a pool of multi-potent progenitors for lamellocytes specification in case of parasitisation. It is believed that the JAK/STAT signalling pathway is modulated upstream by Col and Notch in the PSC in a non-cell autonomous manner (Krzemien et al., 2007; Mandal et al., 2007).

On the other hand, lamellocytes are absent in healthy larvae under normal conditions but the differentiation will be rapidly initiated when parasitic infection

with eggs of wasps such as *Leptopilina heterotoma* or *Leptopilina boulardi* is detected (Rizki and Rizki, 1992). It is believed that the PSC releases an as yet unidentified signal to the medullary zone that is able to suppress the JAK/STAT signalling pathway via a short type 1 cytokine receptor Latran and hence allowing the massive differentiation of prohemocytes into lamellocytes to subsequently be released into the hemolymph to encapsulate the wasp egg that is too big to be phagocytosed by plasmatocytes (Krzemien et al., 2007; Makki et al., 2010). It has also be shown that during lamellocyte differentiation upon immune challenge, crystal cell differentiation has been abolished 24 hours after the parasitisation suggesting that it is likely a common hematopoietic progenitor that gives rise to both lamellocytes and crystal cells (Krzemien et al., 2010b).

1.2.2.1.3 Haematopoiesis Beyond Larval Stages

The lymph gland begins to degenerate and release its content into the circulation from early pupa stages. The majority of hemocytes in the primary lobes are released in the first five hours post puparium formation and by 12 hours the primary lobes will become empty. At the same stage, the secondary lobes also begin to dissociate following a rapid, but not complete, period of prohemocytes differentiation into plasmatocytes. By 15 hours after puparium formation, the lymph gland is completely empty of hemocytes, begins to diminish and finally disappears (Grigorian et al., 2011; Lanot et al., 2001). The crystal cell and lamellocyte populations also appear to be abolished shortly after the larva enters the puparium stage. Crystal cells can still be detected two hours after the onset of metamorphosis but the population of cells is lost by six hours later (Grigorian et al., 2011). Lamellocytes have only been observed when the larva is parasitised and

hence its presence has never been detected in healthy larvae. In adult, no hematopoietic organ has yet been identified. The majority of adult hemocytes are sessile and tissue associated and are composed of both embryonic and larval origin as shown by transplantation assay (Holz et al., 2003). Plasmatocytes are the major hemocyte population that accounts for 95% of mature blood cells in larvae and the only known existing blood cells in adult fly.

1.2.2.2 Functions of Hemocytes

1.2.2.2.1 Phagocytosis and Encapsulation

One of the major functions of *Drosophila* hemocytes is phagocytosis, including removal of damaged, dying or excess cells, microbes that are potentially a threat to the fly and foreign bodies (Stuart and Ezekowitz, 2008). Plasmatocytes are the most abundant hemocyte population and also the key cell type responsible for the process. This population of hemocytes has been described as being analogous to mammalian monocytes/macrophages. It is capable of phagocytosing both apoptotic corpses and dead cellular material throughout different developmental stages as well as microbes upon infection. During embryogenesis, plasmatocytes are able to take up cells or tissues that are undergoing apoptosis during the process of morphogenesis, to eliminate damaged cells and to control cell numbers in the growing embryo. For instance, the shaping of the tracheal system and the formation of the central nervous system (Baer et al., 2010; Sears et al., 2003). During metamorphosis, plasmatocytes are responsible for taking up and recycling doomed cells as well as clearing the degenerating lymph gland (Grigorian et al., 2011; Lanot et al., 2001). Removal of dead cellular material is mediated by the

scavenger receptor, Croquemort, a homologue to the mammalian scavenger receptor CD36 (Franc et al., 1996; Franc et al., 1999; Tepass et al., 1994).

In addition to engulfing apoptotic bodies, plasmatocytes are also involved in immune surveillance upon infectious challenge. Plasmatocytes are responsible for phagocytosing invading bacteria and fungi to prevent spreading and further infection. Absence of plasmatocytes caused by specific targeted apoptosis or genetic depletion results in a strong susceptibility to infections. The reduced resistance to both bacterial and fungal infection results from faster outgrowth of pathogens not kept in check by being engulfed by plasmatocytes (Charroux and Royet, 2009; Defaye et al., 2009). Infection with microbes that are too big to be internalised by plasmatocytes will stimulate the maturation of lamellocytes (Rizki and Rizki, 1992). Lamellocytes are a population of hemocytes that appear to be large, flat, adherent and their differentiation only take place after the larval immune system is provoked by parasitoid infection (Carton and Nappi, 1997; Rizki and Rizki, 1992). Following maturation in the lymph gland, lamellocytes migrate to the site of infection and adhere to the surface of the parasite forming a multi-layered, melanotic capsule that eventually kill the parasite by impairing oxygen supply or by production of cytotoxic free radicals, quinones or semiquinones (Nappi et al., 1995, 2000).

1.2.2.2.2 Production of Cytokines and Modulation of the Systemic Immune Responses

Besides the strong phagocytic capacity, *Drosophila* hemocytes are a source of cytokine production in response to infection and are able to influence the systemic immune responses in larval stages. The linkage between the cellular and humoral immune responses is first demonstrated by the observation that the humoral immune response can be inhibited by impairing the phagocytic activity of hemocytes (Elrod-Erickson et al., 2000). In Imd mutant flies, co-injection of polystyrene beads and *E. coli* enhances the defect of humoral immunity in Imd mutant flies. The death of the phagocytosis-impaired flies is accelerated and only 5% of flies are able to survive more than three days compared to 60% survival rate in flies with normal phagocytic capacity, suggesting hemocytes act in concert with the humoral immune response upon immune challenge (Elrod-Erickson et al., 2000). Integration between cellular and humoral immunity was further characterised in adult flies. Following septic injury, Unpaired 3 (Upd3) expression is highly upregulated in hemocytes, but not in the fat body (Agaisse et al., 2003). The *Drosophila* Upd3 is a secreted cytokine that is most related to the mammalian IL-6 and binding of Upd3 to its receptor Dome triggers the downstream JAK/STAT signalling pathway. In response to septic injury, hemocytes produce and secrete Upd3 into the hemocoel, and it binds to the Dome receptors expressed on the fat body. Activation of the JAK/STAT pathway in the fat body results in the transcription of target genes totA and Tep1, showing that hemocytes play a signalling role upon immune challenge and are important in integrating the cellular and systemic immune responses (Agaisse and Perrimon, 2004; Agaisse et al., 2003).

Significant expression of several members of the Toll pathway, including Toll itself, Cactus, Pelle, MyD88 and Tube, with particularly high levels of Spz, is observed in all populations of larval hemocytes, (Irving et al., 2005). Importantly, Spz RNA production is constitutive in the fat body without the presence of infection. Spz is also strongly induced in hemocytes – but not in the fat body – after an immune challenge. A disruption in the production of Toll-dependent AMPs by the fat body was also observed in hemocyte-depleted larvae. In the absence of hemocytes, Drosomycin expression by the fat body tissue is severely downregulated following *M. luteus* infection, hence confirming the production of Spz by hemocytes is a target of microbial pattern receptors and reaffirming the role of larval hemocytes in activating the systemic immunity upon immune challenge (Irving et al., 2005; Shia et al., 2009).

Besides Upd3 and Spz, subsets of adult *Drosophila* hemocytes have also been shown to express two TGF-β superfamily members, dawdle (daw) and decapentaplegic (dpp) as a consequence of bacterial infection and wounding (Clark et al., 2011). The expression of daw in response to wounding is regulated by the Toll pathway. The key role of daw is to suppress melanisation by inhibiting serine protease 7 (Sp7), a major regulator of infection-induced melanisation, expression (Castillejo-Lopez and Hacker, 2005). Overexpression of daw following *Listeria monocytogenes* infection leads to rapid death through severe suppression of Sp7-induced melanisation. On the other hand, dpp is induced by infection and injury via activation of the JNK pathway. In the absence of infection, dpp suppresses expression of antimicrobial peptides to prevent unnecessary AMPs responses (Clark et al., 2011).

In conclusion, hemocytes have been demonstrated to play a crucial role in integrating the cellular and humoral immune responses under infectious and inflammatory conditions. Detection of damage signals and/or microbial components by a yet unidentified mechanism in 3rd instar larvae triggers production of cytokines by hemocytes that are able to prime the fat body for JAK/STAT- or Toll-dependent stress protein or AMPs induction, respectively. In adult *Drosophila*, specific populations of hemocytes also display the ability to modulate immune responses. Following injury and infection, expression of members of the TGF-β superfamily via activation of the Toll and JNK pathway regulates melanisation and AMPs responses, hence modulating survival and wound healing. The capability of hemocytes to integrate the two responses in a systemic reaction not only represents a new paradigm of *Drosophila* immunity, but also shows a strong correlation to the mammalian immune response making *Drosophila* an excellent model to study the complex regulations of innate immune system mediated by monocytes/macrophages. (Fig. 1-4)

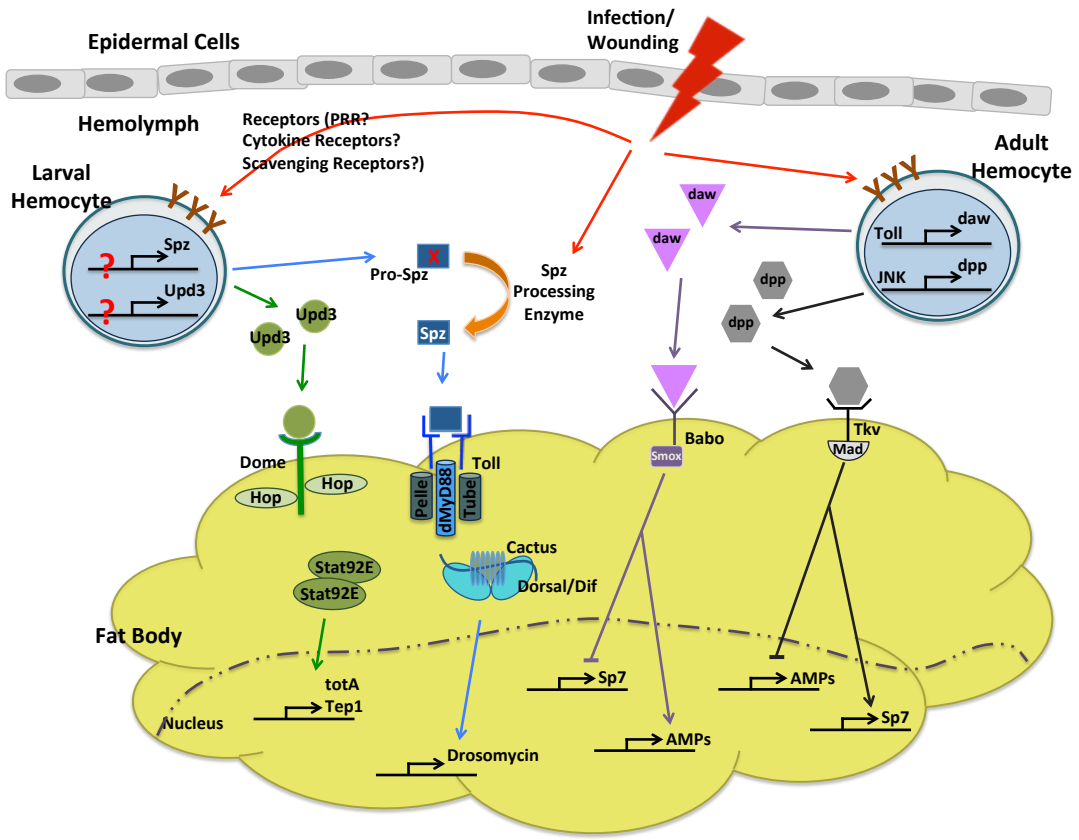


Figure 1-4: ***A new paradigm of Drosophila immunity – integration of the cellular and humoral immune responses by hemocytes.*** In response to infection and/or tissue damage, larval hemocytes are able to produce and secrete cytokines Spz and Upd3 into the hemolymph that activates the downstream signalling cascade in the fat body. In adult *Drosophila*, specific subsets of hemocytes are capable of expressing two members of the TGF-β superfamily – daw and dpp – following infection and wounding. Activation of the downstream pathway of daw and dpp leads to suppression of serine protease Sp7 to inhibit melanisation and AMPs responses, respectively.

1.3 Questions, Aims and Experimental Approach

The aim of this project is to gain more insights into the transcriptional regulation of cytokine genes in *Drosophila* hemocytes and to consequently identify novel positive and/or negative regulators that might be involved in the regulation of corresponding cytokines production by mammalian monocytes.

The immune system of *Drosophila* and mammals share many common features. The intracellular signalling pathways activated upon infectious challenge are highly conserved between the fly and the mammalian innate immune responses. The *Drosophila* Toll pathway closely resembles the mammalian TLR-1 pathway, whereas the Imd pathway has shown similarity to the mammalian TNF signalling pathway. Recent studies on *Drosophila* hemocytes have demonstrated that a specific population of fly hemocytes – the plasmatocytes – is able to integrate the cellular and the humoral immune responses through secretion of cytokines in response to immune cues including tissue damage and microbial components. This novel finding has demonstrated a new potential in using *Drosophila* hemocytes to assess the complex processes employed to regulate cytokine transcription in human and mouse monocytes.

In this project, we are aiming to study cytokine regulation in hemocytes on transcription level during infection and inflammatory conditions. We initiated the project with three *Drosophila* cytokines – Spätzle, Unpaired3, and Eiger, but eventually narrowed down solely to Spätzle. It is believed that inactive pro-Spz, produced by the fat body, circulates in the hemolymph during normal status. Of

note, a recent study based on a genome wide screen revealed that Spz is also constitutively expressed by the fat body tissue without the presence of immune stimuli (Irving et al., 2005). In order to follow and study Spz transcription, we have engineered a Spz reporter fly in which Spz expression is reported through green fluorescent protein (GFP) in a Gal4-UAS independent manner. In this Spz reporter, GFP expression will be driven and regulated by the 5' and 3' homology regions (3kb long each) directly cloned from the Spz endogenous locus. By imaging this reporter fly with confocal microscopy and performing immunostaining in dissected tissues, we were able to establish a profile reflecting Spz expression during different developmental stages at steady state – embryonic, larval and adult. In order to precisely follow the endogenous expression of Spz, we also attempted to generate knock-in reporters by replacing one of the Spz alleles with GFP. However, the GFP intensity of this reporter is too low to be detected. Nevertheless, flies with both Spz alleles replaced by GFP is a Spz null mutant that allows us to study the immune responses to fungus and Gram-positive bacteria invasion in the absence of Spz.

Since Spz expression is elevated in larval hemocytes after immune challenge, we thus aim to further interrogate the transcription regulation of Spz specifically in hemocytes during infection and inflammatory conditions. In order to follow *Drosophila* hemocytes, we have generated hemocyte reporters that enable us to trace hemocyte expression. Hemocytes are labelled with red fluorescence proteins (RFP) using dual transgenic constructs based on the Gal4-UAS expression system imported from yeast. The system consists of a transcription factor (Gal4) and its target sequence (UAS). Expression of Gal4 is driven by a hemocyte specific

reporter - we use Singed (Sn-Gal4 construct) and Croquemort (Crq-Gal4 construct) - while a second transgene drives expression of the fluorescent reporter under UAS repeats (UAS-RFP).

By crossing the Spz reporter with the hemocyte reporter, it allows us to follow Spz expression specifically in hemocytes during the steady state and under challenged conditions. To verify that the reporter flies function correctly, we will challenge such flies with wounding and infectious agents to induce Spz production, and monitor eGFP expression in hemocytes in anesthetised flies, in vivo, as a function of time. We will study kinetics of GFP expression, the numbers of cytokine producing cells, and their distribution in the fly in relation with the wounded/infected area. (Fig. 1-5) Depending on results, we will prepare a screen for quantitative regulators of cytokine transcription in hemocytes, using cell-specific gene knockdown in hemocytes by RNAi. Libraries of UAS-RNAi flies will be purchased from Japanese and Austrian consortiums, and crossed with the reporter flies. Because the reporter flies contain a Crq-Gal4 construct, F1 flies will specifically express the RNAi – and silence expression of genes – in hemocytes. To validate the strategy, we will initially test the hypothesis that silencing Toll or other components in the signalling pathway, will down-regulate infection-inducible Spz expression by hemocytes.

Although this project is focusing on Spz alone, the system will be applicable to other *Drosophila* cytokines (Unpaired3 and Eiger) once it is validated and hence provide us a great potential to thoroughly understand the transcriptional regulation of cytokines in hemocytes in challenged status.

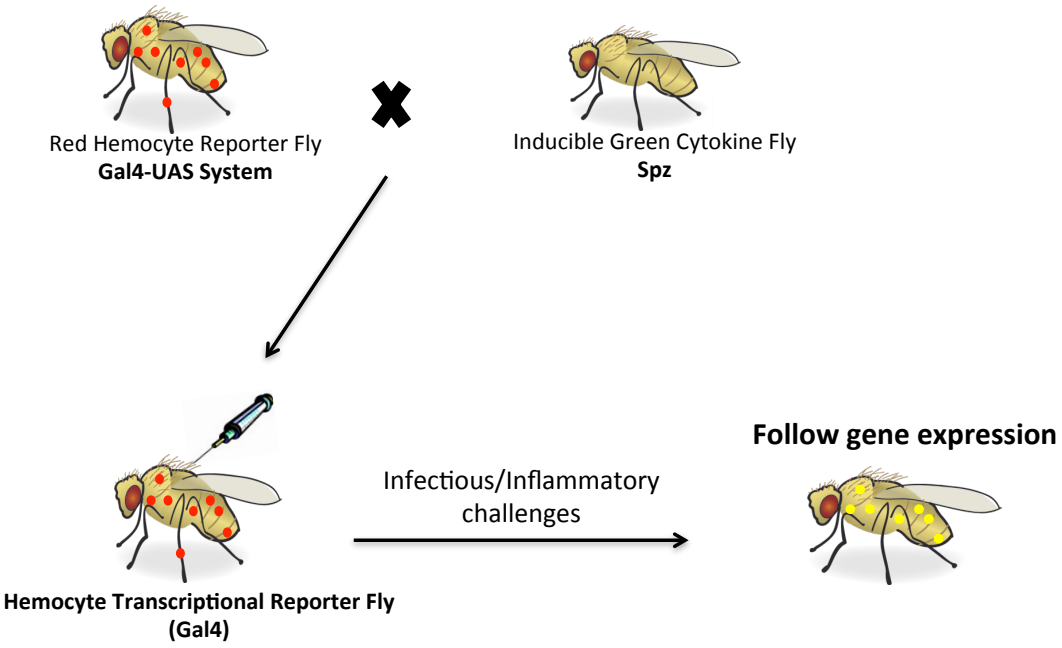


Figure 1-5: ***Schematic illustration for the generation of the Hemocyte Transcriptional Reporter Fly.*** In order to follow Spz transcription in hemocytes during different immune conditions, Hemocyte reporter is crossed with Spz reporter in which hemocytes are labelled by RFP using the Gal4-UAS expression system while Spz expression is represented by GFP. By following GFP signals specifically in hemocytes post-infectious challenge using fluorescent or confocal microscopy, it enables us to study the kinetics of Spz transcription in hemocytes.

- Chapter 2 -
Material and Methods

2.1 Drosophila Strains and Crosses

2.1.1 Fly Strains and Stocks

All fly stocks were maintained at 18°C on a medium consisting of 8% Fructose, 10% Yeast, 2% Polenta and 0.8% Agar. Fly strains used are listed below:

Wild Type		Oregon-R
		w^{1118}
Hemocyte Driver		w;;Crq-Gal4/TM6c,Sb^1 (Crq)
Strains with Fluorescent Protein		w;UAS-nls-dsRed
		w;UAS-RedStinger (Bloomington 8546)
		w;;UAS-CD8-mCherry (Bloomington 27398)
		w;Sn-Gal4,UAS-RedStinger/Cyo (Brian Stramer's Lab, KCL)
Chromosome Balancer		w;noc^{Sco}/SM6a
		w;;TM2/TM6c,Sb^1
		w;$noc^{[Sco]}$/Sm6a;TM2/TM6c,Sb^1
		w;IF/Cyo;MKRS/TM6b,Sb^1
Spz-eGFP Transgenic Flies	Line 1	w;;Spz-eGFP/TM6c,Sb^1
	Line 2	w;;Spz-eGFP/TM6c,Sb^1
	Line 3	w;Spz-eGFP/SM6a
	Line 4	w;;Spz-eGFP/TM6c,Sb^1
	Line 5	w;;Spz-eGFP/TM6c,Sb^1
Spz-neGFP Transgenic Flies	Line 1	w;Spz-neGFP
	Line 2	w;Spz-neGFP/SM6a
	Line 3	w;;Spz-neGFP
Spz-2xeGFP Transgenic Flies	Line 1	w;Spz-2xeGFP
	Line 2	w;;Spz-2xeGFP/TM6c,Sb^1
Spz knock in Reporter		w;;$Spz^{eGFP/TM6c,Sb'}$ (Heterozygous knock in) or w;;$Spz^{eGFP/eGFP}$ (Homozygous knock in/Knock out, abbreviated as Spz^{eGFP})
hs-FLP, hs-I-SceI Flies		yw/w;hs-FLP,hs-I-SceI/Cyo (Bloomington 6934)

2.1.2 The Gal4-UAS Expression System

In order to visualise *Drosophila* hemocytes and other tissues, the Gal4-UAS system was applied in conjunction with various fluorescent proteins. The Gal4-UAS system, which provides gene and tissue specific expression using the yeast transcription factor Gal4, and the corresponding Upstream Activation Sequence (UAS) (Brand and Perrimon, 1993). Transcription of Gal4 is controlled by the upstream promoting region from the gene of interest and binding of the Gal4 to its responding element UAS results in transcriptional activation of the downstream genetic material (Fig. 2-1).

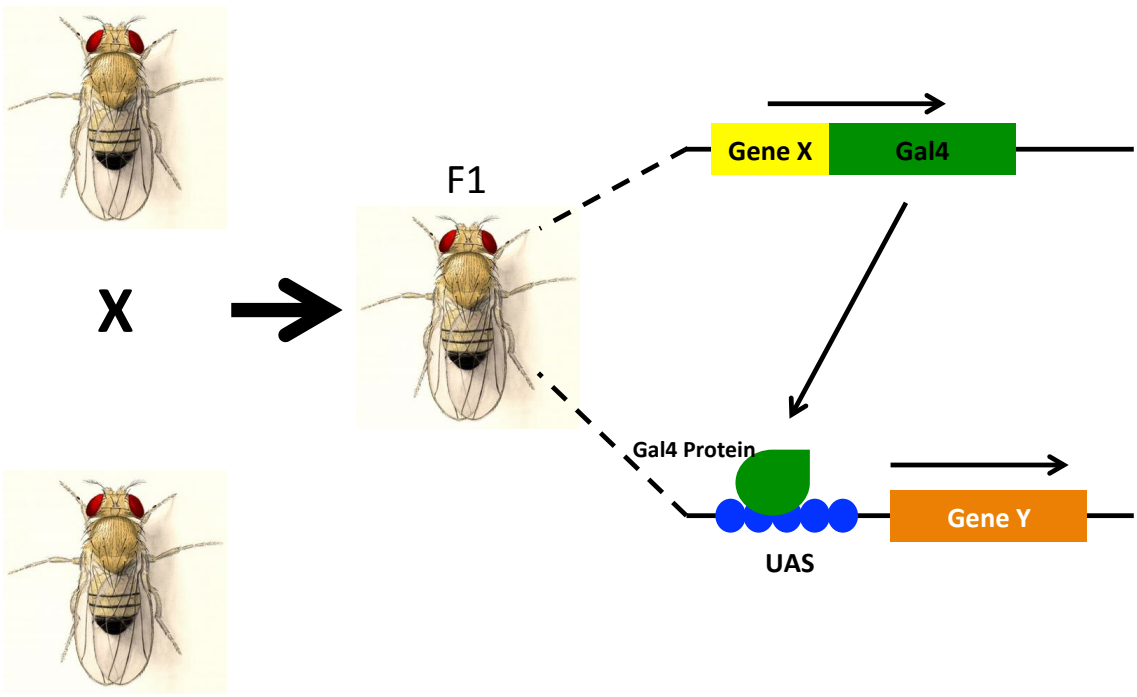


Figure 2-1: ***Schematic illustration of the Gal4-UAS expression system.***

To follow hemocytes at different development stages, various hemocyte reporters were used. During embryo development, *Singed-Gal4* was used (Sn-Gal4,UAS-

RedStinger/Cyo, obtained from Brian Stramer lab) to report embryonic hemocytes (Barolo et al., 2004; Zanet et al., 2009a; Zanet et al., 2009b). During larval and adult stages, *Croquemort-Gal4* (Crq-Gal4) (Franc et al., 1996; Franc et al., 1999) was used to trace hemocytes with two different types of RFP (w;;Crq-Gal4, UAS-CD8-mCherry/TM6c,Sb[1]; w;UAS-nls-dsRed/SM6a;Crq-Gal4/TM6c,Sb[1]).

2.1.3 Fly Crosses

Fly crosses for generating different reporters were carried out in standard 25mm diameter vials, with six to nine virgin females (♀) and three to five males (♂). Flies for virgin collection were kept at 18°C and virgins were collected twice per day while crosses were kept at 25°C. All flies for crosses were between 5 to 10 days old. Male flies for bacteria or fungi infection were transferred into fresh medium and kept at 25°C 24 hours prior to injection and post injection. A maximum of 20 flies were kept in one vial and all flies were between 7 to 15 days old.

2.1.3.1 Hemocyte Reporter

Cross Scheme for $\mathbf{w;;\frac{Crq-Gal4,UAS-CD8-mCherry}{TM6c,Sb'}}$

P_0 ♀♀ $\mathbf{w;;\frac{Crq-Gal4}{TM6c,Sb'}}$ x **w;;UAS-CD8-mCherry** ♂♂

↓

F_1 ♀♀ $\mathbf{w;;\frac{Crq-Gal4}{UAS-CD8-mCherry}}$ x $\mathbf{w;;\frac{TM2}{TM6c,Sb'}}$ ♂♂

↓

F_2 ♀♀ $\mathbf{w;;\frac{TM2}{TM6c,Sb'}}$ x $\mathbf{w;;\frac{Crq-Gal4,UAS-CD8-mCherry}{TM6c,Sb'}}$ ♂

Cross scheme for w; $\frac{\text{UAS−nls−dsRed}}{\text{SM6a}}$; $\frac{\text{Crq−Gal4}}{\text{TM6c,Sb′}}$

P$_0$ ♀♀ **w; $\frac{\text{noc[Sco]}}{\text{SM6a}}$; $\frac{\text{Crq−Gal4}}{\text{TM6c,Sb′}}$** x ***w; UAS − nls − dsRed*** ♂♂

F$_1$ ♀♀ **w; $\frac{\text{UAS−nls−dsRed}}{\text{SM6a}}$; $\frac{\text{Crq−Gal4}}{+}$** x **w; $\frac{\text{noc[Sco]}}{\text{SM6a}}$; $\frac{\text{TM2}}{\text{TM6c,Sb′}}$** ♂♂

F$_2$ ♀♀ **w; $\frac{\text{UAS−nls−dsRed}}{\text{SM6a}}$; $\frac{\text{Crq−Gal4}}{\text{TM6c,Sb′}}$** x **w; $\frac{\text{UAS−nls−dsRed}}{\text{SM6a}}$; $\frac{\text{Crq−Gal4}}{\text{TM6c,Sb′}}$** ♂♂

2.1.3.2 Spz-Hemocyte Reporter

Cross scheme for w; $\frac{\text{Spz−neGFP}}{\text{SM6a}}$; $\frac{\text{Crq−Gal4,UAS−CD8−mCherry}}{\text{TM6c,Sb′}}$

P$_0$ ♀♀ **w; Spz − neGFP** x **w; ; $\frac{\text{Crq−Gal4,UAS−CD8−mCherry}}{\text{TM6c,Sb′}}$** ♂♂

F$_1$ ♀♀**w; $\frac{\text{Spz−neGFP}}{+}$; $\frac{\text{Crq−Gal4,UAS−CD8−mCherry}}{+}$** x **w; $\frac{\text{noc[Sco]}}{\text{SM6a}}$; $\frac{\text{TmM2}}{\text{TM6c,Sb′}}$** ♂♂

F$_2$ ♀♀ **w; $\frac{\text{Spz−neGFP}}{\text{SM6a}}$; $\frac{\text{Crq−Gal4,UAS−CD8−mCherry}}{\text{TM6c,Sb′}}$** x **w; $\frac{\text{Spz−neGFP}}{\text{SM6a}}$; $\frac{\text{Crq−Gal4,UAS−CD8−mCherry}}{\text{TM6c,Sb′}}$** ♂♂

2.2 Construction of the Cytokine-GFP Plasmids

2.2.1 Drosophila Genomic DNA Extraction

Flies were anaesthetised with CO_2 and individual was picked and mashed with 50µl Squashing Buffer (10mM Tris-Cl pH8.2, 1mM EDTA, 25mM NaCl, and 200µg/ml Proteinase K), followed by incubation at 37°C for 30 minutes, then for 2 minutes at 95°C to inactivate the Proteinase K. The DNA was kept at -20°C for long-term storage.

2.2.2 Amplification of 5' and 3' Homology Region (HR) of Spz, Upd3 and Egr

The Expand Long Template PCR System kit (Roche, Ref no.: 11681834001) was used to amplify the 3kb long fragment upstream and downstream of the coding sequence of *Spz, Upd3 and Egr*. Genomic DNA extracted from a male wild type fly (w^{1118};;;) was used as a template. Designated restriction sites had been inserted (underlined) into the fragments during PCR with the primer sequences shown as follow:

For *Spz*

Primer set A

For Spz-5' HR (*Not*I, *Spe*I)

Forward primer (5'):

TAGGGCGGCCGCACGCACATATTAACAGGTATTCAAAGCTTTAC

Reverse primer (3'): TAGGACTAGTGCTCGATCTCGGGCTTAATC

Primer set B

For Spz-3'HR (*Asc*I, *BsiW*I)

Forward primer (5'): TAGGGGCGCGCCTTCTGGAAAATGGGATTCTCCAGTCG

Reverse primer (3'): TAGGCGTACGCCGTAGATTGCATCTACACGTCAC

For *Upd3*

Primer set C

For Upd3-5'HR (*NotI*, *Acc65I*)

Forward primer (5'): TAGGGCGGCCGCCCAGCGACCT

Reverse primer (3'): TAGGGGTACCCTGTGGATGGAA

Primer set D

For Upd3-3'HR (*AscI*, *BsiWI*)

Forward primer (5'): TAGGGGCGCGCCAAGAGGGTCGAT

Reverse primer (3'): TAGGCGTACGACGAGAGCAGAGAG

For Egr

Primer set E

For Egr – 5'HR (*NotI*, *Acc65I*)

Forward primer (5'): TAGGGCGGCCGCGACAAGCAACAA

Reverse primer (3'): TAGGGGTACCAATTGATGAACACTGGGG

Primer set F

For Egr – 3'HR (*AscI*, *BsiWI*)

Forward primer (5'): TAGGGGCGCGCCGCACGCACACAC

Reverse primer (3'): TAGGCGTACGATTGGAGAGATTATCCCCGG

PCR Cycle

94°C	2min		
94°C	10sec	┐	
50°C	30sec	10 cycles	
68°C	8min	┘	
---	---	---	---
94°C	15sec	┐	
50°C	30sec	25 cycles	
68°C	8min*	┘	*Add extra 20sec per cycle
68°C	10min		
4°C	∞		

2.2.3 Amplification of eGFP

eGFP with designated restriction sites inserted (underlined) into both ends was amplified using PCR, from the plasmid pTub-eGFP using primers shown below:

Primer set G

eGFP-Set 1 (*BamH*I, *Pst*I)

Forward primer (5'): GATTGGATCCATGGTGAGCAAGGGCG

Reverse primer (3'): GATTCTGCATGTACAGCTCGTCCATG

Primer set H

eGFP-Set 2 (*Nco*I, *Sal*I)

Forward primer (5'): GATTCCATGGATGGTGAGCAAGGGCG

Reverse primer (3'): GATTGTCGACCTTGTACAGCTCGTCCATG

PCR Cycle

94°C	5mins		
94°C	30sec	┐	
50°C	30sec		35 cycles
72°C	1min	┘	
4°C	∞		

All PCR products were electrophoresed on an ethidium bromide-stained 1% agarose gel and purified using QIAquick Gel Extraction kit (Qiagen, Cat no.: 28704).

2.2.4 Cloning

All cloning was performed using the TaKaRa DNA Ligation Kit LONG (Takara Bio Inc., Cat no.: 6024) (Osoegawa *et al.*) and generated vectors were transformed into either One Shot® TOP10 Chemically Competent *E. coli* (Invitrogen, SKU: C4040-03) or XL 10-Gold® Ultracompetent Cells (Stratagene, Cat no.: 200315). Isolated colonies were grown overnight in a 37°C shaking incubator in LB broth

(Invitrogen, Ref no.: 12795-084) with ampicillin (100μg/ml), the plasmids were harvested and extracted by using QIAquick Spin Miniprep Kit (Qiagen, Cat no.: 27106). Successful insertion was confirmed by performing colony PCR using Set G eGFP primers and digestions with appropriate enzymes (NEB). All PCR products and diagnostic digestions were run on 1% agarose gel.

2.2.5 Plasmid Sequencing

To confirm the correct sequence of eGFP and IRES, the 2xeGFP plasmid was sequenced before cloning into the pW25-*Spz* 5' construct. The eGFP-IRES-eGFP fragment from the 2xeGFP plasmid was first amplified with T3 and T7 primers by PCR supplemented with BigDye. The PCR product was then purified with ethanol precipitation. Purified sample was sent out for the sequencing procedure performed by a commercial company Geneservice. All sequencing data was analysed with software ApE.

PCR Cycle

96°C	30sec	┐	
50°C	15sec		30 cycles
60°C	60sec	┘	
20°C	∞		

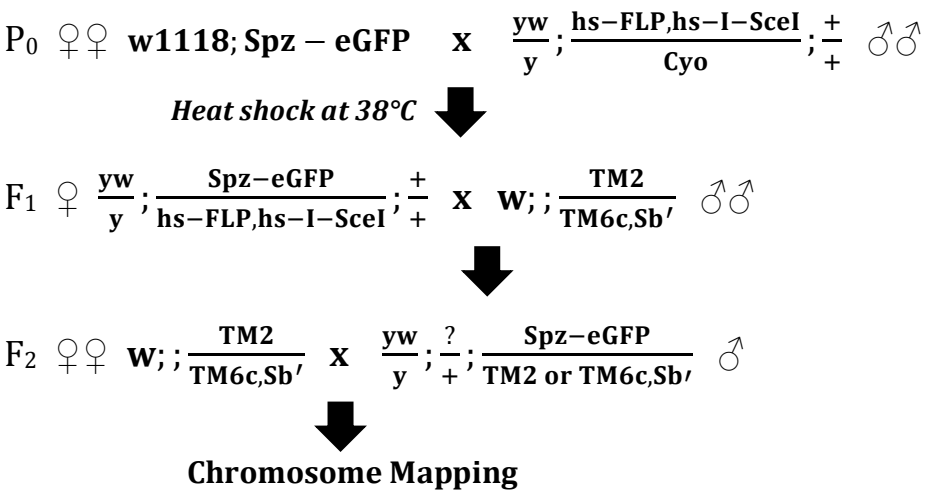
2.3 Generation of Transgenic and knock in (KI) Spz Reporter Flies

2.3.1 Transgenic Insertion

The transgenic insertions were performed by a commercial company BestGene Inc. The Spz-eGFP, Spz-2xeGFP, and Spz-neGFP plasmids were shipped in ddH_2O with 1mM EDTA. The plasmids were transformed and purified by miniprep upon arrival and mixed with helper plasmid supplied by BestGene Inc. prior to microinjection into 200 w^{1118} embryos. The surviving G_0 adults were back-crossed individually to w^{1118} expanded by crossing G_1 to w^{1118} and only G_2 transformants were shipped back. Upon receiving the G_2 transformants, the chromosomal location of the plasmid was mapped and balanced with chromosome balancer lines to establish a stable stock.

2.3.2 knock in (KI) Crosses

The cross scheme shown below was described by Huang et al. and was used to generate the Spz-KI reporter from the Spz-Transgenic reporter (Huang et al. 2008). Only transgenic flies with a pW25-Spz-GFP plasmid insertion into the 2^{nd} chromosome were used for the KI crosses. Shown below are crosses for generating the Spz-eGFP KI reporter. The same process was used for the generation of Spz-2xeGFP and Spz-neGFP KI reporters.



(Huang et al., 2008)

After eggs were laid in the food, parents were removed from the vial and the eggs were being heat shocked for 30 minutes to 1 hour in a circulating water bath set at 38°C. The water level should be higher than the food in the vial but lower than the cotton plug. After heat shocking, the vials were returned to 25°C incubator to continue development.

Successful insertion of the Spz-KI fragment into the Spz locus was first screened by the eye colour (Further discussed in Chapter 4.2). After selecting the orange eye flies, individual fly were crossed with 3rd chromosome balancer to map out the chromosomal location of the Spz-KI fragment and further confirmed by long range PCR and PCR.

2.3.3 Genotyping

Long range PCR and PCR were performed to confirm a successful KI process. Genomic DNA extracted from the homozygous Spz-KI reporter was used as a template and the primer sequences used are listed below:

Primer set I

5'HR – Long Range PCR, Set 1

Forward primer (5'): GACCACTTGGACACACATG

Reverse primer (3'): GAACTTCAGGGTCAGCTTG

Primer set J

5'HR – Long Range PCR, Set 2

Forward primer (5'): CGCAAAGTTGTGCTAATATGATAC

Reverse primer (3'): TGAACAFCTCCTCGCCCTT

Primer set K

3'HR – Long Range PCR, Set 1

Forward primer (5'): TTCGCTGCATGAATTAGCTTG

Reverse primer (3'): CAATGTTCGGAACTCCCAAT

Long Range PCR Cycle

94°C	2min		
94°C	10sec	┐	
50°C	30sec	10 cycles	
68°C	5min	┘	
---	---	---	---
94°C	15sec	┐	
50°C	30sec	25 cycles	
68°C	5min*	┘	*Add extra 20sec per cycle
68°C	10min		
4°C	∞		

PCR Cycle

94°C	5mins		
94°C	30sec	┐	
50°C	30sec	35 cycles	
72°C	2mins	┘	
4°C	∞		

All PCR products were run on an ethidium bromide-stained 1% agarose gel.

2.3.4 RNA Collection and Spz and GFP Protein Transcript Level Quantification by Real time-PCR

Three age-matched (<7 days old) male adult flies of each genotype (w^{1118};;;, heterozygous Spz-eGFP KI reporter, and homozygous Spz-eGFP KI reporter) were collected for RNA extraction with Trizol (Invitrogen, Cat no.: 10296-010). RNA was then treated with DNase I (Fermentas, Cat no.: EN0521), and used in a reverse transcription reaction with M-MuL V Reverse Transcriptase (Fermentas, Cat no.: EP0352) for cDNA synthesis. For Real time PCR, 2µl of cDNA was mixed with 5µl of Sensi-Mix™ SYBR Green (Biolone, Cat no.: QT 650-05) and 0.2µl of 10µM primers to set up a 10µl reaction. Transcript levels were detected by a Corbett Roto-Gene 6000 and were normalised to Rpl1 mRNA values. Primer sequences used are shown below:

Spz
Forward primer (5'): GGACGACACCTGGCAGTTA
Reverse primer (3'): GGGTTGATCCGCTCCTTC

GFP
Forward primer (5'): AGTCCGCCTGAGCAAAGA
Reversed primer (3'): TCACGAACTCCAGCAGGACC

Rpl1
Forward primer (5'): TCCACCTTGAAGAAGGGCTA
Reverse primer (3'): TTGCGGATCTCCTCAGACTT

2.3.5 Emergence Rate Quantification

To quantify the emergence number of female and male KI reporter (w;;Spz$^{eGFP/Tm6c,Sb'}$), flies were backcrossed with w^{1118} (cross scheme as shown below). The initial cross was expanded to 20 tubes and the number and sex of the hatching flies were recorded.

P_0 ♀♀ **w^{1118};;;** **x** **w;;Spz$^{eGFP/Tm6c,Sb'}$** ♂♂

↓

F_1 ♀ **w;;Spz$^{eGFP/+}$** **x** **w;;Spz$^{eGFP/+}$** ♂♂

↓

Fly Counting

2.4 Infection and Survival Experiments

2.4.1 Bacteria and Fungi Preparation

E. coli and *M. luteus* cultures were grown shaking in LB broth overnight at 37°C and collected by centrifugation at 4°C at 1600rpm for 10 minutes the following morning. The absorbance of bacteria was measured at the wavelength of 600nm (OD_{600}). The *E. coli* and *M. luteus* cultures were first mixed in equal quantities and the mixture for injection was diluted to 1 at OD_{600} with sterile PBS (Invitrogen, Cat no.: 14190-169).

C. albicans (SC 5314) (From Dr. Julian Naglik, Dental Institute, King's College London) cultures were grown overnight in YPD liquid medium (1% Yeast Extract, 2% Peptone, and 2% Glucose) in a 30°C shaking incubator. The cultures were centrifuged at 4°C for 10 minutes and the pellet was washed twice with sterile ice-cold PBS. The absorbance was measured at OD_{600} and diluted to 1 with sterile PBS before injecting into flies.

2.4.2 Injection

Flies for injection were anesthetised with CO_2 and injected with a pulled glass capillary needle (Needle puller: Narishige, Model PC-10) and a Picospritzer® III. For each condition, injections were calibrated individually by measuring the diameter of the expelled drop under a pot of oil consisted of mineral oil (Sigma, Product no.: M8410-500mL) and Halocarbon oil 700 (Sigma, Product no.: H8898-100mL) so that 50nl of bacteria mixture was injected into flies.

2.4.3 Survival Experiment

Groups of 20 adults, age 4 to 7 days old, were collected and treated with designated experimental stimulus. After challenge, the flies were transferred into a new vial immediately and incubated at 25°C throughout the whole period. The numbers of dead flies were counted twice a day and flies were transferred into a fresh vial every other day to prevent stuck down on the food due to high humidity in the vial. Any death recorded within the first 3 hours after immunisation was not included in the analysis.

2.5 Imaging

All images were taken by a SP5 confocal microscope using LAS-AF software purchased from Leica. Images were taken by a 10x (NA 0.4), 20x Dry (NA 0.5), or 40x Oil (NA 1.25) lens with the resolution of 1024x1024 pixels, laser speed of 400Hz for live larvae/flies and 200Hz for processed tissues, with line average of four. Images were processed and analysed with imaging software Imaris (Version 7).

2.5.1 In-vivo Imaging of Embryos

For imaging purposes, embryos were collected from a grape juice agar plate installed at the bottom of the embryo collection chamber the previous evening. Embryos were then washed with bleach to remove chorion. Embryos at appropriate stages were picked using a dissecting microscope and immobilised on a stage made with plastic membrane with low-melt agarose. A cover slip was placed on top of the embryos with support from two cover slips that were put on each side of the embryos to avoid damage.

2.5.2 In-vivo Imaging of Larvae

Individual larva was picked directly from the medium and washed with ice-cold PBS. A single clean larva was then immobilised on a 24x60mm microscope cover glass using double-sided tape. A small piece of damp tissue was used to surround the larva to keep it moist during imaging. A cover slip was placed on top of the larva with support from tape that was folded into the same height as the larva.

While imaging, the cover glass was placed upside down so that the laser could come through the clear cover slip.

2.5.3 In-vivo Imaging of Adult Drosophila

Individual adult fly was anesthetised with CO_2 and stuck down on a 24x60mm microscope cover glass on the dorsal side by superglue (Loctite). During imaging, flies were kept anesthetised with CO_2 supplied directly from a CO_2 pipe.

2.6 Drosophila Dissection, Tissue Preparation, and Immunostaining

2.6.1 Larva Dissection

Wandering third instar larvae were picked from medium and washed with ice-cold PBS. Clean larvae were then placed on a Silicon Pad for dissection. The larvae were kept under 20µl of ice-cold PBS during dissection and were opened at the posterior end with a pair of fine micro-scissors. By holding the larval head with a pair of fine forceps, it was rolled inside out carefully on the tip of the forceps to expose the inside of the larva and the larva was fixed immediately.

2.6.2 Adult Dissection

Four to seven days old adult males were picked and anesthetised. Each fly was wetted with isopropanol before soaking in PBS. The head was removed with a pair of fine micro-scissors before the whole intestine was pulled out from the fly body but grabbing the posterior end with a pair of fine forceps. The fly body and intestine were fixed immediately and for the adult fat body, the fly body was opened after an overnight fixation and the fat body tissues were carefully scraped away from the cuticle.

2.6.3 Immunostaining

Dissected larval tissues and adult intestine were fixed immediately with 4% formaldehyde (Thermo Scientific, Product no.: 28908) in PBS at room temperature for 30 minutes on a rocker whilst the body of an adult fly was fixed with 4% formaldehyde in PBS at room temperature overnight on a rocker. Fixed samples

were then transferred into an eppendorf prefilled with 1ml PBST (PBS + 0.1% Triton® X-100 [Sigma, Product no.: X100-500mL]), followed by four 5-minute washes with PBST at room temperature on a wheel. Following a 30 minutes permeabilisation in PBS with 0.5% Triton® X-100 for 30 minutes at room temperature, samples were washed once with PBST for 5 minutes and pre-blocked with 10% Normal Goat's Serum (NGS) or 0.5% Bovine Serum Albumin (BSA) in PBST for 1 hour at room temperature. After blocking, samples were incubated with primary antibody (1:150, Polyclonal Rabbit Anti-GFP, Invitrogen, Ref. no.: A11122) in 10% NGS or 0.5% BSA in PBST overnight at 4°C in the dark and washed five times (5 minutes each) with PBST. Samples were then incubated with secondary antibody (1:150, Goat Anti-rabbit 488, Invitrogen, Ref. no.: A11034) in 10% NGS or 0.5% BSA in PBST for 4 hours at room temperature, followed by nuclear staining with DAPI (1:1000) in PBST for 10 minutes at room temperature and four 5 minute washes with PBST. Processed larvae or tissues were then mounted with Vectashield (Vector, Lot no.: X0301) and kept at 4°C in the dark.

- Chapter 3 -
Generation of Cytokine knock in Plasmids

3.1 Introduction

In respond to infection or immune challenge such as wounding, corresponding cytokines will be stimulated and subsequently trigger the downstream signalling pathway to protect the host. On the other hand, *Drosophila* hemocytes play an important role in senescing pathogens as well as secreting cytokines like Spätzle (Spz) and Unpaired3 (Upd3) upon immune challenge. Nonetheless, little is known about the regulation of these cytokines in genetic level in hemocytes and hence requires further studies to understand better about the transcriptional regulations of these cytokines in these cells following immune challenge. To accomplish this aim, we are developing a genome wide screening system using reporter flies that contain fluorescently labelled cytokine (Spz, Upd3 or Egr) and hemocytes. By silencing genes specifically in hemocytes with different RNAi lines, we will be examining the effect of these genes on cytokine transcription by assessing changes of the cytokine level using confocal microscopy. The genome wide screen will not only allow us to gain further insight into the genetic regulation of individual cytokine, it also helps up to identify new potential upstream regulator of each cytokine. In this screening system, the knock down effect of RNAi lines will be tissue specific and driven by a Hemocyte-Gal4 reporter. Thus, it will require a fluorescent cytokine reporter that is independent on the Gal4-UAS expression system. Therefore, we have engineered new knock in cytokine reporter by inserting green fluorescent protein into the coding region of each cytokine using a knock in plasmid. Details on the production of the knock in plasmids are reported in this chapter.

3.2 Amplification of the 5' and 3' Homology Regions

To generate a knock in reporter flies, we will be using the ends-out gene targeting technique described previously by Huang et al. (Huang et al., 2008). This technique will allow us to replace the coding region of each cytokine by green fluorescent protein and hence enable us to measure endogenous production of each cytokine using fluorescent microscopy. Generation of knock in reporter flies require a knock in construct composing of the vector pW25, a 3kb long upstream and downstream homology region of each cytokine, and green fluorescent protein. Long range PCR was performed with specifically designed primers to amplify the homology regions upstream and downstream of *Spz*, *Upd3* and *Eiger* (*Egr*) (Fig. 3-1A, Fig. 3-4A, Fig. 3-5A) gene coding region directly from genomic DNA. Each PCR fragment is up to 3kb long and a unique restriction site had been inserted into both ends of each fragment through long range PCR for cloning purpose. For primer set used to amplify the 5' homology region of *Spz*, a *Not*I and *Spe*I sites were inserted at the 5' and 3' end, respectively, whereas a *Not*I and *Acc65*I sites were inserted correspondingly at the 5' and 3' end of both the 5' homology region of *Upd3* and *Egr*. For the 3' homology region, a *Asc*I site was inserted at the 5' end of this region of all cytokine, whilst a *BsiW*I site was inserted at the 3' end of the 3' homology region of each cytokine. Following confirmation of the correct sequence of the 5' and 3' homology regions by diagnostic digestions with several restriction enzymes, the PCR products were digested with restriction enzymes corresponding to the restriction sites inserted.

3.3 Generation of pW25-Spz-GFP knock in constructs

Three variants of pBluescript-*Spz* 5'-eGFP constructs were made with different number(s) and forms of eGFP: an eGFP construct with a single eGFP, a 2xeGFP construct with two eGFPs, and a construct with nuclear eGFP (neGFP), to ensure strong GFP intensity for fluorescent imaging. Two amplicons of eGFP with different restriction sites were amplified by PCR using an eGFP-containing pTub plasmid as template (Fig. 3-2A), while neGFP was directly excised out of a pH-Stinger plasmid (Fig. 3-2B). The 3kb long 3' homology region of *Spz* was first inserted into the knock-in plasmid pW25 between the *Asc*I and *BsiW*I restriction sites (Fig. 3-2C), while the 3kb long 5' homology region of *Spz* was cloned into the vector pBluescriptKS+ (pBS) using restriction sites *Not*I and *Spe*I (Fig. 3-2D). Successful cloning was confirmed by diagnostic digestion with designated restriction enzymes (Fig. 3-2C' and 3-2D').

3.3.1 Spz-eGFP knock in Construct

To generate the pW25-*Spz*-eGFP construct, a single eGFP was cloned into the pBS-*Spz* 5' plasmid between restriction sites *BamH*I and *Pst*I to generate a pBS-*Spz* 5'-eGFP construct (Fig. 3-3A). The *Spz* 5'-eGFP fragment was excised from the pBS-*Spz* 5'-eGFP construct by restriction digestion with *Not*I and *Kpn*I, followed by cloning of the Spz 5'-eGFP fragment into the pW25-Spz 3' constructs between *Not*I and *Acc65*I, with *Kpn*I from the Spz 5'-eGFP fragment compatible with the Acc65I site (Fig. 3-3A). The final knock-in construct was confirmed by diagnostic digestions using *BamH*I and *Pst*I (Fig. 3-3A').

3.3.2 Spz-2xeGFP knock in Construct

As the GFP intensity of a single eGFP might not be strong enough for imaging purpose, we have decided to engineer a construct that contains two eGFPs and thus enhance the fluorescent intensity. For the pW25-*Spz*-2xeGFP construct, a 2xeGFP construct was first generated using pBS. Following insertion of eGFP into *EcoR*V and *Sal*I of pBS, a Cricket Paralysis Virus (CrPV) IRES was cloned into the pBS-eGFP plasmid between *EcoR*I and *Nco*I, with the second eGFP cloned into *BamH*I and *Pst*I sites (Fig. 3-3B). To ensure that the multiple digestions and cloning processes did not induce mutation, especially in the eGFP-IRES-eGFP area, the plasmid was sequenced. After confirming no mutation in the 2xeGFP fragment with sequencing data, the 2xeGFP fragment was then excised from pBS between *BamH*I and *Kpn*I sites and replaced eGFP in the pW25-*Spz*-eGFP constructs with these restriction sites (Fig. 3-3C). Successful insertion of 2xeGFP into pW25-*Spz* plasmid was first screened by colony PCR for eGFP (Fig.3-3C' top panel) and was followed by a diagnostic digestion with EcoRV (Fig. 3-3C' bottom panel).

3.3.3 Spz-neGFP knock in Construct

The neGFP fragment from the pH-Stinger plasmid was cloned into the pBS-*Spz* 5' construct between *BamH*I and a blunted *Cla*I site. To generate the pW25-*Spz*-neGFP construct, the neGFP fragment was cut from the pBS-*Spz* 5'-neGFP plasmid at *BamH*I and *Kpn*I sites. The eGFP in the pW25-Spz-eGFP constructs was excised and was replaced in the same location by the neGFP fragment (Fig. 3-3D). To confirm successful generation of the pW25-*Spz*-neGFP construct, the plasmid was screened by colony PCR for eGFP (Fig. 3-3D' top panel) followed by a diagnostic digestion with *Not*I (Fig. 3-3D' bottom panel).

3.4 Generation of pW25-Upd3-eGFP and pW25-Egr-eGFP knock in construct

To generate the pW25-*Upd3*-eGFP knock in construct, the *Upd3* 5' homology region was first blunted at both ends and cloned into a pBS vector using the *Not*I and *Acc65*I restriction sites (Fig. 3-4C) while the 3' homology region was cloned into the pW25 plasmid between *Asc*I and *BsiW*I sites (Fig. 3-4B). Following successfully cloning of the 5' homology into pBS, a single eGFP was cloned into the pBS-*Upd3* 5' plasmid between restriction sites *BamH*I and *Pst*I (Fig. 3-4C). The Upd3-eGFP fragment was excised from the pBS-Upd3 5'-eGFP construct by restriction digestion with *Not*I and *Acc65*I, before cloning of this fragment into the pW25-Upd3 3' plasmid between these two sites. Several attempts were made for the last cloning (*Upd3* 5'-eGFP fragment into pW25-*Upd3* 3') but no positive clone was picked (Fig. 3-4D).

For pW25-Egr-eGFP knock in construct, the cloning process was identical to the cloning process for generation of pW25-Upd3-eGFP KI construct. Blunted Egr 5' homology region and 3' homology region were first cloned into pBS and pW25, respectively. Successfully cloning of the 3' homology region into pW25 was confirmed by restriction digestion with selected restriction enzymes *Asc*I and *BsrG*I (Fig. 3-5B). However, no positive clone was selected from the cloning of 5' homology region into pBS and thus no further cloning progress was made.

3.5 Discussion and conclusion

We have successfully generated three Spz-GFP knock in constructs with three variants of GFP – eGFP, 2xeGFP (eGFP-IRES-eGFP) and neGFP. Generation of these cytokine knock in constructs involves a complex cloning strategy with a series of cloning. Thus, construction of these knock in plasmid was the most time consuming stage during the development of the genome wide screen. In our original plan, the screen will be used to study the transcriptional regulation of three *Drosophila* cytokines – Spätzle, Unpaired3 and Eiger. But due to the extensive time consumption and the low efficiency in cloning large fragments, we have decided to focus only on generating Spz knock in reporter flies using the three available Spz knock in constructs. Hence, the direction of the screen will be changed to solely study the transcriptional regulation of Spz in hemocytes upon immune challenge.

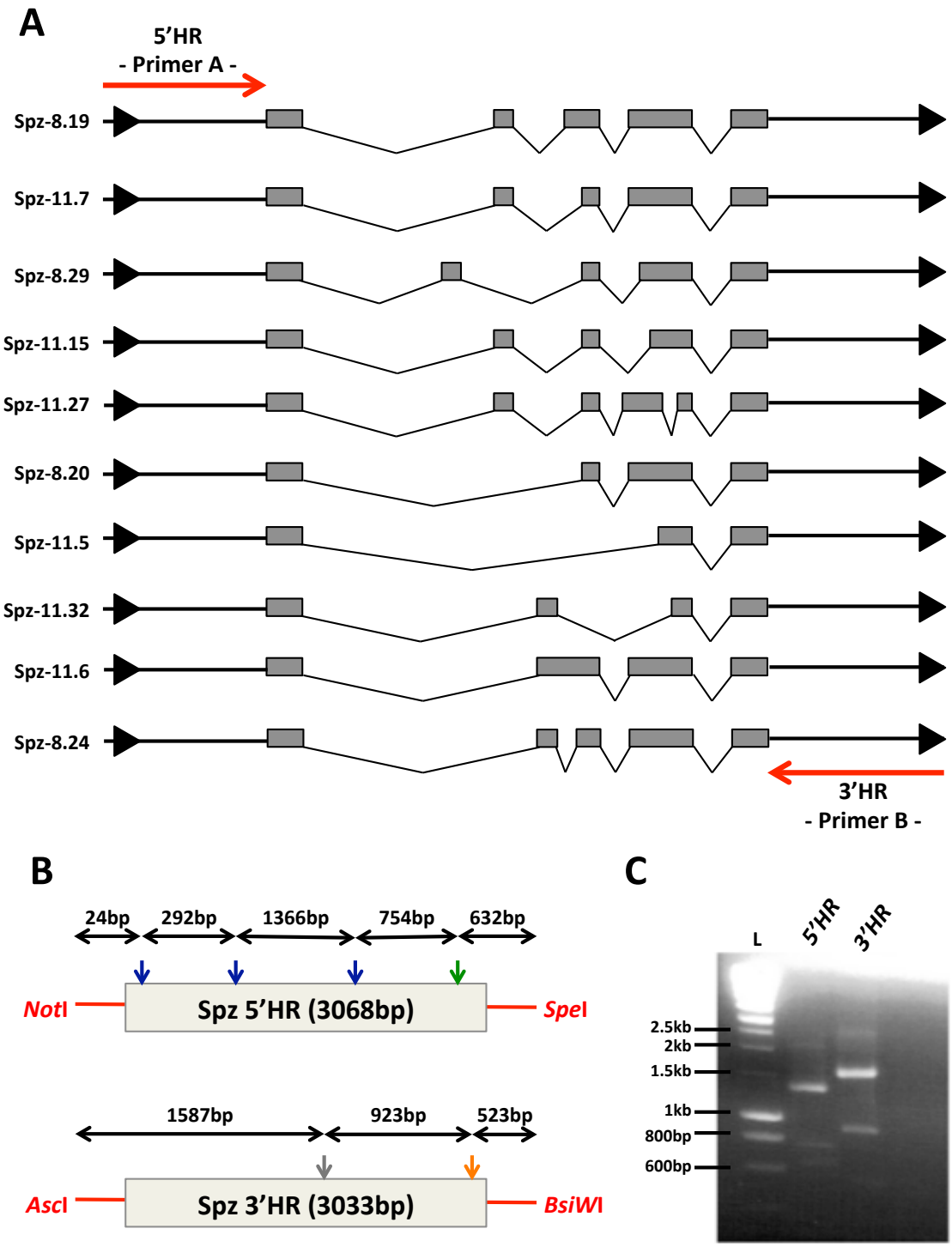


Figure 3-1: ***Amplification of Spz 5' and 3' homology regions (HR) by long range PCR.***

(A) PCR amplification of *Spz* 5' and 3' homology regions (HR) using primer set A and B, respectively. Each PCR fragment is about 3kb long with designated

restriction sites inserted at each end of the fragment. Each diagram represents an isoform of *Spz* as a result of alternative splicing (DeLotto et al., 2001). The diagrams include introns and exons (grey boxes) are not shown to scale.

(B) Specific restriction sites were inserted into the 5' and 3' end of each homology region through long range PCR. The PCR products were digested with appropriate restriction enzymes to confirm the correct sequence. The 5' homology region was digested with *Hind*III (restriction sites indicated by blue arrow) and *Xho*I (restriction site indicated by green arrow) and the expected bands size with the correct sequence will be 24bp, 292bp, 754bp and 1366bp, as shown in (C) lane 5'HR. The 3' homology region was digested with *Spe*I (restriction site indicated by grey arrow) and *Nco*I (restriction site indicated by orange arrow) to give three bands with size of 523bp, 923bp and 1587bp, as shown in (C) lane 3' HR.

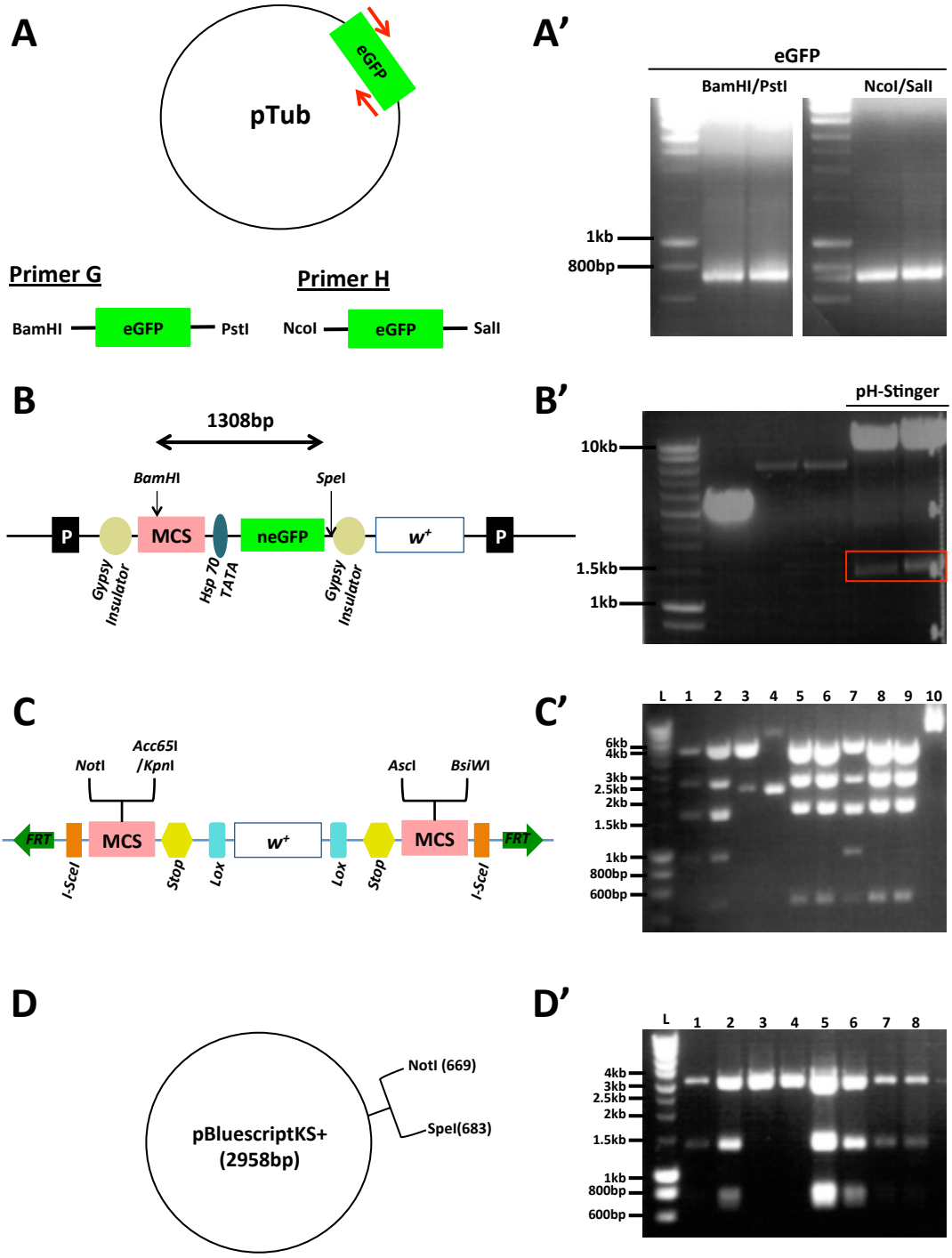


Figure 3-2: ***Generation of various forms of eGFP and cloning of Spz 5' and 3' HRs.***

(A) Amplification of eGFP by PCR using a pTub plasmid as template. Two sets of eGFP with different specific restriction sites inserted into the 5' and 3' end of the eGFP fragments were amplified using two sets of specifically designed primers

(Primer C and D), and the size of the fragments are 717bp (A'). The red arrows indicate the locations where the 5' and 3' primers bind in the plasmid.

(B) neGFP was obtained by digesting a pH-Stinger plasmid with restriction enzymes *BamH*I and *Spe*I and the size of the neGFP fragment is 1308bp (circled in red) (B').

(C) The 3'HR of *Spz* was cloned into the knock-in plasmid pW25 between restriction sites *Asc*I and *BsiW*I. Potential clones were picked and grown and successful insertion was confirmed by diagnostic digestion with *Nco*I and *Spe*I and the band sizes were expected to be 473bp, 923bp, 1598bp, 1692bp, 2585bp and 4716bp (C').

(D) The *Spz* 5'HR was cloned into pBS between the *Not*I and *Spe*I sites. A diagnostic digestion with *Hind*III and *Xho*I was performed and successful insertion was confirmed by band sizes of 668bp, 754bp, 1366bp and 2911bp (D').

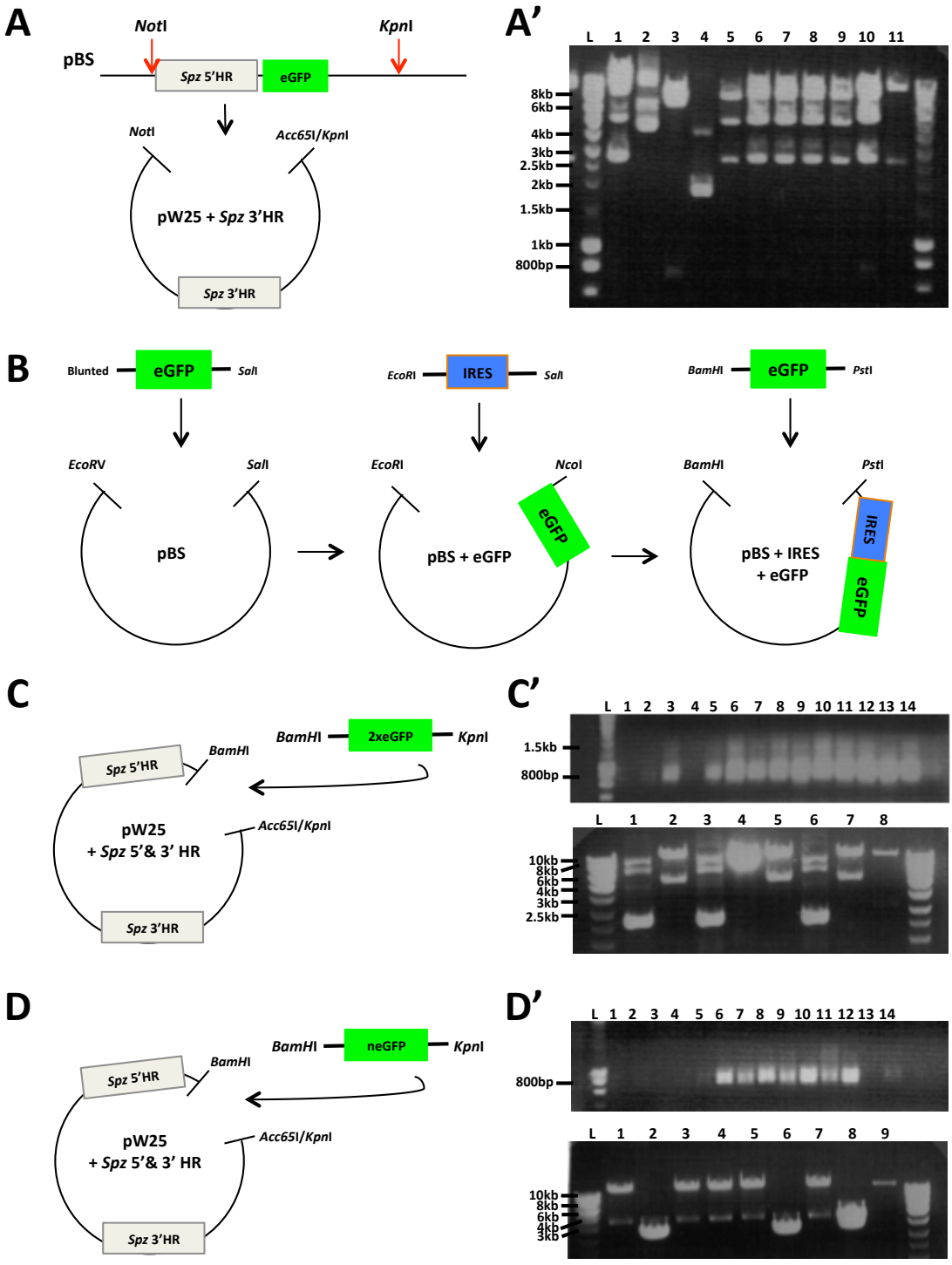


Figure 3-3: ***Cloning strategies for various forms of pW25-Spz-eGFP plasmids.*** (A) To construct the pW25-*Spz*-eGFP plasmid, *Spz* 5'-eGFP fragment was cloned into the pW25-*Spz* 3' plasmid after the fragment was cut out from the pBS plasmid at *BamH*I and *Kpn*I sites. (A') Diagnostic digestion with *BamH*I and *Pst*I was

performed and the expected band sizes for successful insertion are 21bp, 727bp, 2610bp, 4753bp and 7735bp.

(B) To generate 2xeGFP, eGFP with *Nco*I and *Sal*I insertion was first cloned into pBS plasmid with the *Nco*I blunted and preserved for IRES insertion. A viral IRES was cloned into the pBS-eGFP construct using *EcoR*I site from the pBS plasmid and the *Nco*I site from the eGFP fragment. Last, another eGFP fragment with *BamH*I site and *Pst*I site was cloned into the pBS plasmid forming the 2xeGFP construct.

(C) For the pW25-*Spz*-2xeGFP construct, 2xeGFP was excised and cloned into the pW25-*Spz* plasmid using *BamH*I and *Kpn*I sites. Potential clone(s) with 2xeGFP insertion was first screen by colony PCR using Primer C to amplify the 717bp eGFP (C' Top Panel) and positive clones were then digested with *EcoR*V, which cuts the pW25-*Spz*-2xeGFP plasmid twice producing bands with sizes of 5353bp and 11412bp, to confirm correct insertion (C' Bottom Panel).

(D) The pW-*Spz*-neGFP was generated by insertion neGFP fragment into pW25-*Spz* plasmid using *BamH*I and *Kpn*I sites. Successful insertion was confirmed by diagnostic digestion with restriction enzyme *Not*I, producing two bands of 4143bp and 12008bp (D' Bottom Panel), following a positive colony PCR with Primer C amplifying the 717bp eGFP (D' Top Panel).

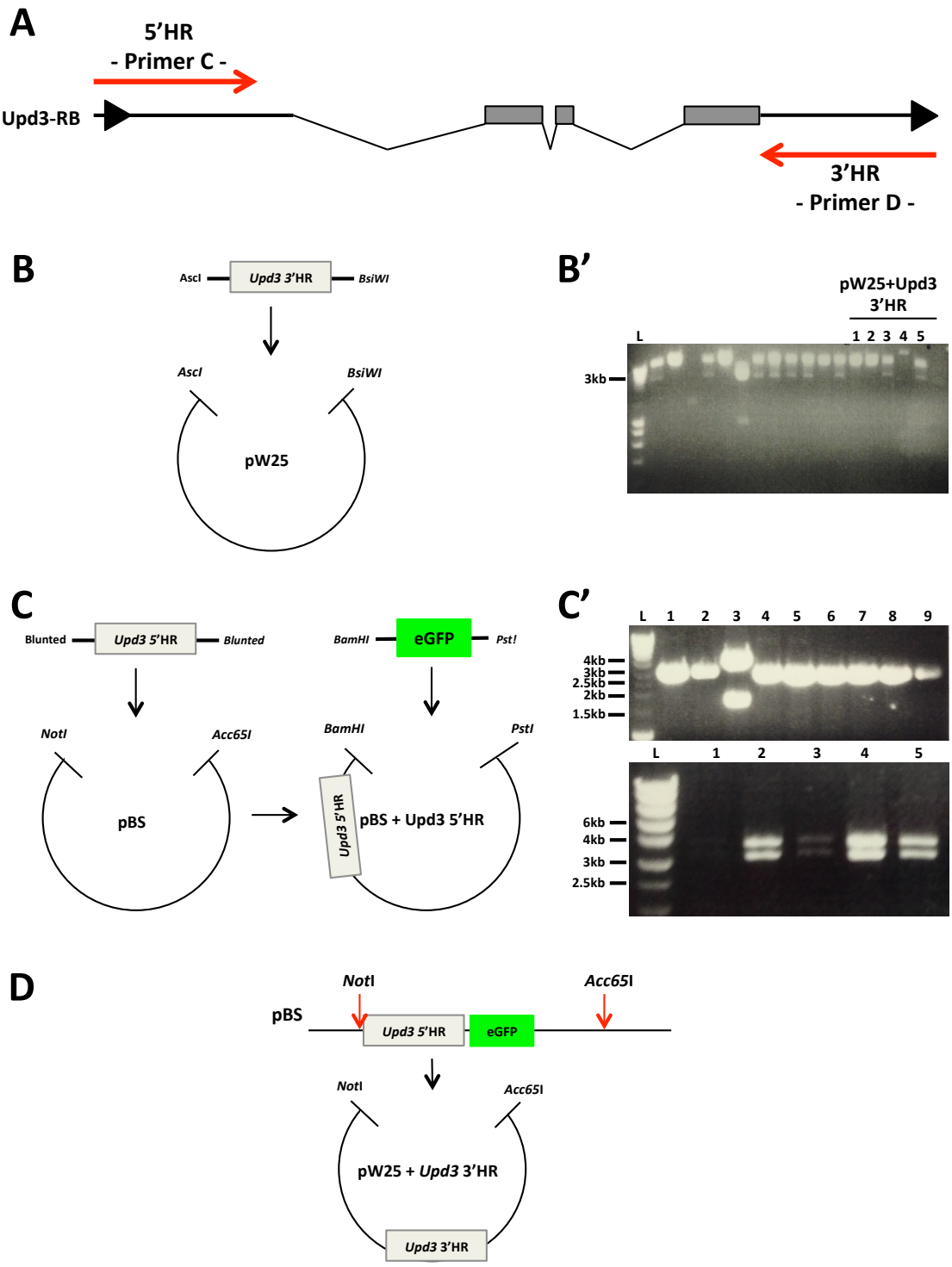


Figure 3-4: ***Cloning strategy for pW25-Upd3-eGFP plasmid.***

(A) PCR amplification of *Upd3* 5' and 3' homology regions (HR) using primer set C and D, respectively. Each PCR fragment is about 3kb long with designated

restriction sites inserted at each end of the fragment. The diagram includes introns and exons (grey boxes) are not shown to scale.

(B) Cloning of the *Upd3* 3'HR into the pW25 plasmid between *AscI* and *BsiWI* sites. (B') Diagnostic digestion with *AscI* and *BsiWI*. Two bands of 8968bp and 3221bp were expected with successful insertion of the Upd3 3'HR into pW25.

(C) Cloning of the *Upd3* 5'HR and eGFP fragment into plasmid pBS. The *Upd3* 5'HR was first blunted at both end and cloned into the pBS plasmid between *NotI* and *Acc65I* followed by cloning of the eGFP fragment between *BamHI* and *PstI*. (C') Diagnostic digestion with restriction enzyme *EcoRI* to confirm successful cloning of *Upd3* 5'HR into pBS (Upped panel). With insertion and correct orientation, three bands of 105bp, 1777bp and 4197bp were expected. Successful cloning of eGFP into pBS-*Upd3* 5'HR was confirmed by digestion with restriction enzyme *AleI* and five bands of 69bp, 150bp, 177bp, 3099bp and 3263bp were expected (Bottom panel).

(D) Cloning of the *Upd3* 5'HR-eGFP fragment into pW25-*Upd3* 3'HR plasmid to generate pW25-*Upd3*-eGFP knock in plasmid. However, no successful clones were detected.

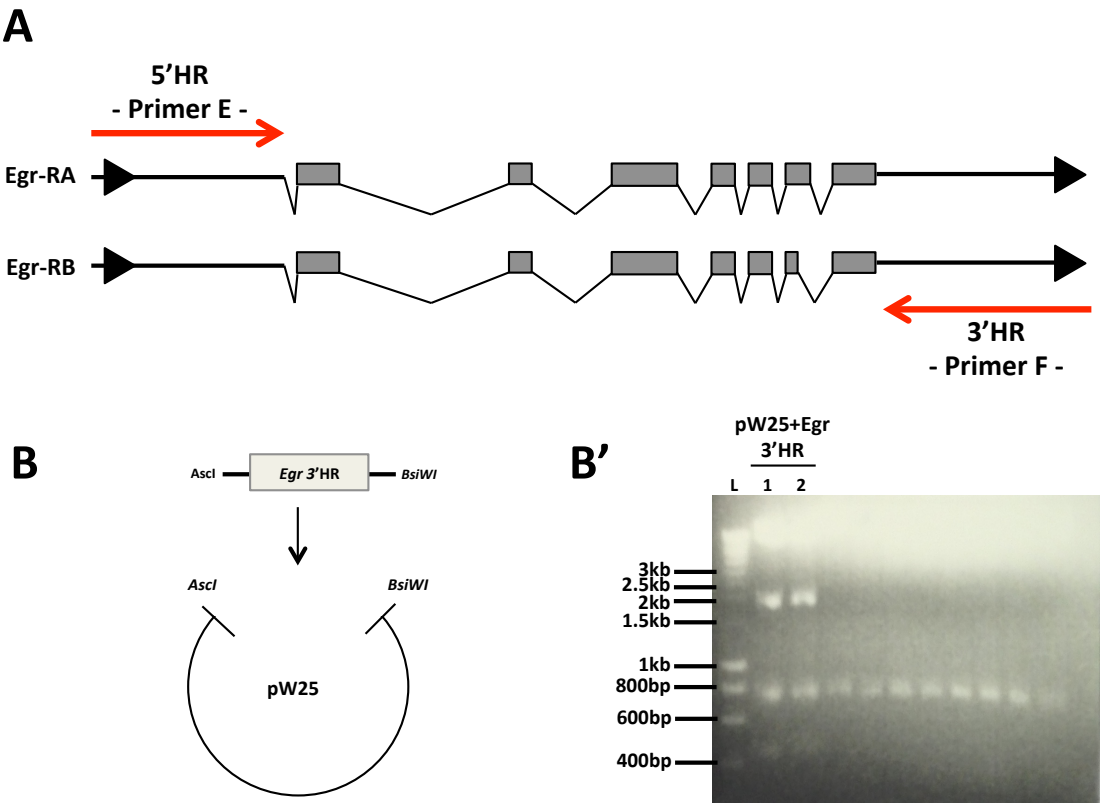


Figure 3-5: ***Cloning strategy for pW25-Egr plasmid.***

(A) PCR amplification of *Egr* 5' and 3' homology regions (HR) using primer set E and F, respectively. Each PCR fragment is about 3kb long with designated restriction sites inserted at each end of the fragment. Each diagram represents an isoform of *Egr* as a result of alternative splicing. The diagram includes introns and exons (grey boxes) are not shown to scale.

(B) Cloning of the *Egr* 3'HR into the pW25 plasmid between *AscI* and *BsiWI* sites.

(B') Successful cloning was confirmed with diagnostic digestion with restriction enzyme *BsrGI* resulting in five bands of 437bp, 735bp, 759bp, 1901bp and 8262bp.

- Chapter 4 -
Generation and Phenotypic Characterisation of *Spätzle* knock in Reporter

4.1 Introduction

After successful cloning of the pW25-Spz-eGFP, pW25-Spz-neGFP and pW25-Spz-2xeGFP plasmids, the knock in plasmids were first inserted into the fly genome on a random basis before further knock in processes as described previously (Huang et al., 2008). We have successfully generated a Spz knock in fly (Spz^{eGFP}/TM6c,Sb^{1}) with the pW25-Spz-eGFP plasmid and conducted phenotypic characterisations. We were unable to detect green fluorescent signal from this knock in fly using confocal microscopy or immunostaining with anti-GFP antibodies in steady state. Nevertheless, the homozygous Spz^{eGFP} knock in reporter is actually a Spz knock out and it is the first and only known existing Spz null mutant. Phenotypic characterisations and survival assays post bacteria and fungi infection had been conducted on the Spz null mutant. Base on preliminary results, flies are more susceptible to bacteria infection and a significant reduced survival following immune challenge with fungi in the absence of Spz.

4.2 Generation of Spz knock in reporter flies

After generation of the pW25-*Spz*-eGFP, pW25-*Spz*-2xeGFP and pW25-*Spz*-neGFP constructs, the plasmids were ready to be inserted into the fly genome and thus they were sent out to a commercial company BestGene Inc. for embryonic injection. Insertion of genetic material into *Drosophila* genome is performed using transposable elements, mainly by the *Drosophila*-specific class II transposon P-element and corresponding P-transposase, resulting in a random insertion of plasmid into the fly genome. pW25 is a P-element vector originated from the pW35 vector featuring *FRT* recombinase and *I-SceI* endonuclease recognition sites,

together with two *lox* sites located upstream and downstream of the marker gene w^+ (a red eye marker) that are recognised by the Cre recombinase and hence allow removal of the w^+ gene. The pW25-*Spz*-GFP constructs were injected into embryos of w^{1118} background, which has the *white* gene (*w*) mutated giving a white-eye phenotype. Flies that have insertion of the KI construct were screened and selected by their eye colour since the w^+ gene from the pW25 vector will rescue the *white* gene mutation. Transgenic reporter flies were then balanced by crossing to chromosome balancer lines and thus the chromosomal location of the insertion would also be marked out. Only transgenic reporter flies with the KI construct inserted in the second chromosome were used to generate the *Spz* KI reporter flies as the genomic location of the *Spz* gene is on the third chromosome. Using transgenic flies with KI construct inserted in the second chromosome will allow us to distinguish the translocation of the KI fragment from the second chromosome to the endogenous locus of Spz in on the third chromosome. The crossing scheme is shown in Material and Methods (2.3.2) (Huang et al., 2008) (Fig. 4-1).

After mapping the chromosomal location of each KI construct, 3rd instar transgenic larvae were picked and imaged with fluorescent microscopy. Out of the three KI constructs – Spz-eGFP, Spz-2xeGFP, and Spz-neGFP – only the Spz-neGFP KI construct displayed green fluorescent signal at the transgenic stage (Fig. 4-3). Although not all KI constructs show green fluorescent signal at the pre-knock in transgenic stage, all transgenic flies were proceeded for the knock in process as described previously (Huang et al., 2008). Following mating with flies possessing heat-activating flippase activity (yw/y;hs-FLP,hs-I-SceI/Cyo;+/+), F1 female progenies were primarily screened for potential homogenous recombination of the

KI construct with Spz loci according to the eye colour. During homologous recombination, the Spz-KI fragment that contains the W^+ gene for eye colour is cut out from its original insertion site. With the present of the Spz-KI in the genome, the eye colour of the F1 progeny will be orange while white eyes indicate the lost of Spz-KI fragment. Depending on the length of heat shock, the eye colour also displays a mosaic pattern. The longer the heat shock duration, the less pigments remain in the complex eyes (Golic and Lindquist, 1989). With 60 minutes incubation at 37°C, only about 10-30% of eye pigments were seen sporadically on the complex eyes and the density of the pigments were also significantly reduced (Data not shown). Individual virgin female that displayed orange pigments on the eyes was picked and crossed with male 3rd chromosome balancer to locate the chromosomal position of the KI construct. After crossing with male 3rd chromosome balancer to confirm homologous recombination (movement of the KI construct from 2nd chromosome to 3rd chromosome), only stock(s) containing F4 progenies with bright orange (heterozygous for KI construct) or dark red (homozygous for KI construct) eyes were picked for further genotype confirmation with long range PCR. The successful rate of ends-out homogenous recombination is low in *Drosophila*. As reported in Huang et al., the homologous recombination frequency is expected to be around 1/100000 gametes (Huang et al., 2008). In our knock in process, less than 10% females in the F1 progeny displayed mosaicism in the complex eyes.

For the generation of Spz-eGFP KI reporter flies, 2000 virgin females (displayed mosaic eye pigmentation) from the F1 progeny were collected for further crosses and validation. Only 1 stock from the F4 progeny contained purely orange-eyed

offspring giving a successful rate of 0.05%. In addition to phenotype screening, the chromosomal location of the Spz-eGFP construct was further confirmed by long-range PCR using different sets of primers (Fig. 4-2A). The protein transcript level of *Spz* was also measured because in the homozygous knock in reporter ($Spz^{eGFP/eGFP}$, abbreviated as Spz^{eGFP}), the *Spz* coding region on both alleles is replaced by eGFP, and therefore should have no *Spz* expression (Fig. 4-2B).

For the generation of Spz-neGFP and Spz-2xeGFP KI reporter flies, 1000 orange-eyed virgin females were collected from the initial knock in cross but none of the F4 progenies demonstrated a successful homogenous recombination of the KI construct with the Spz locus. Due to limitation of time, we have focused on characterising the Spz-eGFP KI reporter flies.

4.3 Characterisation of the Spz^{eGFP} knock in reporter flies

After confirming knock in of the eGFP into the endogenous *Spz* gene, both Spz^{eGFP}/TM6c,Sb^{1} and Spz^{eGFP} reporter flies were imaged by fluorescent and confocal microscopy in order to characterise the expression of *Spz* reported by eGFP at steady state. Wandering 3rd instar larvae were first picked and imaged by fluorescent microscopy without giving any experimental immunisation but no GFP signal was observed in the KI reporter flies (Fig. 4-4A). Age synchronised (4 to 7 days old) male heterozygous and homozygous knock in flies were collected and imaged by confocal miscopy at steady state (Fig. 4-4B). No GFP signal was observed in heterozygous or homozygous Spz^{eGFP} flies.

Absence of GFP signal in the Spz^{eGFP} reporter flies could be due to two possibilities; the GFP signal was too dim to be detected by the confocal microscopy as only one copy of eGFP was inserted into the *Spz* locus, or a mutation occurred during the cloning procedures and hence the eGFP fragment lost its fluorescence. To address the first possibility that the intensity of the GFP signal was too weak, fat body tissues from the KI larvae (3rd instar) were dissected and fluorescently labelled by anti-GFP antibody since *Spz* is constitutively expressed by the fat body in larvae during steady state to provide an instant response to infection (Irving et al, 2005). However, no GFP signal was detected and observed in the fat body tissue during steady state (Fig. 4-5A) thus indicating that GFP is not expressed in the knock in reporter flies. To further examine the successful production of eGFP in the reporter flies, the transcript level of eGFP was measured by quantitative PCR (Fig. 4-5B). eGFP transcript was detected in both Spz^{eGFP}/TM6c,Sb^{1} and Spz^{eGFP} reporter flies. Homozygous reporter flies transcribed twice as much eGFP compared to the heterozygous reporter flies. However, the level of eGFP transcript in both the homozygous and heterozygous reporter flies was significantly lower than the Spz-neGFP transgenic reporter flies, in which GFP signal was able to be detected and observed using fluorescence microscope and confocal microscopy. Therefore, the Spz^{eGFP} reporter is an inadequate imaging tool.

4.4 Phenotypic characterisation of the Spz^{eGFP} homozygous knock in reporter – a null mutant

Although the Spz^{eGFP} knock in fly is not suitable for imaging, the Spz^{eGFP} reporter is still a useful *Spz* mutant with simple genetic background in comparison with

existing loss of function *Spz* mutants. The *Spz* null mutant is also a useful tool to study the role of *Spz* during embryongenesis and immune responses. The Spz^{eGFP} homozygous knock in flies, in which the zygotic Spz contributions have been removed but not the maternal Spz mRNA, showed no developmental defect and emerged into morphologically normal adults. No significant difference in emergence rate in either sex was observed after backcrossing the heterozygous Spz^{eGFP}/TM6c,Sb^{1} reporter flies to the wild type w^{1118} flies (Cross scheme shown in Material and Methods 2.3.5 and Fig. 4-6A). The Spz^{eGFP} homozygous knock in male flies are fertile and were able to produce healthy progeny when crossed with wild type virgin females whilst Spz^{eGFP} homozygous females are sterile. Eggs were laid by the Spz^{eGFP} reporter females but no larvae hatched from these eggs. The life span of the Spz^{eGFP} homozygous reporter flies was reduced compared to wild type flies but no difference in life span was detected in the Spz^{eGFP}/TM6c,Sb^{1} heterozygous knock in reporter and Spz-neGFP transgenic reporter flies compared to wild type flies (Fig. 4-6B). More than 40% of the Spz^{eGFP} flies died by 21 days after eclosion while all OR and Spz^{eGFP}/TM6c,Sb^{1} heterozygous knock in flies were still alive.

It has been shown that flies are more sensitive to fungal infection in the absence of functioning *Spz* (Lemaitre et al., 1996). Therefore we investigated how the Spz^{eGFP} homozygous knock out mutants respond to infection with fungus and bacteria. Instead of male flies, virgin females were used for this experiment as the emergence rate of Spz^{eGFP} homozygous knock in reporter males was too low from the original stock (Spz^{eGFP}/TM6c,Sb^{1}) – 2/3 less than Spz^{eGFP} homozygous females. Age synchronised (4 to 7 days old) heterozygous virgin females of

Spz^{eGFP}/TM6c,Sb^{1} and homozygous Spz^{eGFP} knock in flies were collected and infected with fungus *C. albicans* (SC 5314) or a mixture of bacteria composed of *M. luteus* and *E. coli*. In this one time experiment, Spz^{eGFP} homozygous knock in flies displayed a reduced life span when the flies were only injected with sterile PBS (Fig. 4-7). 58% of non-treated Spz^{eGFP} homozygous knock in flies survived at Day 21 when only 28% of the mutant flies were able to live after 21 days after PBS injection indicating the lack of *Spz* might contribute to a higher sensitivity to wounding. Moreover, as exhibited previously by Lemaitre and Hoffmann (Lemaitre et al., 1996), loss of *Spz* expression reduces the life span of the flies dramatically after fungal infection. The Spz^{eGFP} hhomozygous reporter also displayed a similar mortality rate as compared to previous description - more than 95% of the Spz^{eGFP} homozygous reporter flies died only 2.5 days after infection with *C. albicans* while 70% of the Spz^{eGFP}/TM6c,Sb^{1} heterozygous knock in flies survived the fungal infection (Fig. 4-8). Nearly 80% of the mutant flies survived until 11 days following infection with *M. luteus* and *E. coli* before the survival declined abruptly from day 13 after exposure to the bacteria and all Spz null mutant flies died on day 20 (Fig. 4-9).

As the emergence rate of male and female homozygous Spz^{eGFP} knock in flies from the original stock that was balanced on the 3rd chromosome (Spz^{eGFP}/TM6c,Sb^{1}) showed a significant difference in comparison to the eclosion rate of homozygous reporter flies that were obtained after backcrossed to w^{1118} (w;;Spz^{eGFP}/+), the survival experiment was repeated using heterozygous Spz^{eGFP}/+ and homozygous Spz^{eGFP} obtained from this cross (Cross scheme as shown in Material and Methods 2.3.5). Age synchronised (4 to 7 days old) males were collected and injected with

sterile PBS or *C. albicans* (SC 5314). As compared to the Spz^{eGFP} homozygous knock in reporters from the original stock, backcrossed homozygous Spz^{eGFP} reporters displayed a prolonged life span in the absence of wounding and infection with 90% of the reporters lived for more than 20 days (Fig. 4-10A). When injected with sterile PBS to injure the flies, homozygous reporters from the backcrossed stock also exhibited a higher survival rate with 65% (Fig. 4-10B) of the flies surviving at day 20 while only 28% of the homozygous Spz^{eGFP} reporters from the balanced stock survived (Fig. 4-9). However, the homozygous knock in flies exhibited a similar survival rate when infected with *C. albicans* compared to the homozygous Spz^{eGFP} knock in flies from the original balanced stock (Fig. 4-11).

These data suggest that presence of the 3rd chromosome balancer (w;;TM2/TM6c,Sb^1) in the parents (Spz^{eGFP}/TM6c,Sb^1) of the homozygous Spz^{eGFP} reporter flies affect the female and male hatching ratio. Significant reduction of male homozygous Spz^{eGFP} reporter was recorded but the reason is unknown. No previous studies shown a role of Spz in determining gender and this imbalance of female/male hatching ration was corrected when backcrossed with w^{118}. Thus, the emergence discrepancy between female and male Spz^{eGFP} homozygous reporter from SpzeGFP/TM6c, Sb^1 stock could be due to the effect of the 3rd chromosome balancer. One the other hand, the life span of the SpzeGFP homozygous reporter flies were slightly affected with the presence of the 3rd chromosome balancer in the parents in the absence of any immune challenge and the sensitivity towards wounding. Nevertheless, it did not alter the resistance towards fungal infection. Taken together, existence of 3rd chromosome balancer in the parents of Spz null mutant could notably alter the female/male emergence rate and slightly reduce the

life span of the null mutant under normal condition. For more accurate results, homozygous Spz^{eGFP} reporter should be obtained from the backcrossed stock to eliminate any possibly affect from the 3rd chromosomal balancer.

4.5 Discussions and conclusion

In order to further understand the genetic regulation of Spz transcription in hemocytes upon activation by different immune stimulus, an *in vivo* genome wide screening is being set up. The genome wide screen will employ a fluorescent Spz reporter that can accurately report the endogenous transcription of Spz and a fluorescent hemocyte reporter. To screen for potential upstream regulators that can modulate Spz transcription in hemocytes, different RNAi lines will be used to specifically knock down target genes in hemocytes and the effect of the gene will be assessed by Spz expression with fluorescence or confocal microscopy.

Using the techniques described in Huang et al. (Huang et al., 2008), we have successfully generated a novel Spz-eGFP knock in reporter out of the three KI constructs (Spz-eGFP, Spz-2xeGFP and Spz-neGFP). In this knock in reporter, eGFP protein is used to replace the coding region of Spz in the 3rd chromosome. Production of eGFP is controlled and regulated by the upstream and downstream regulatory elements of Spz. Through imaging with fluorescence microscopy, it will provide us the most accurate information of Spz expression profile and kinetics during different conditions including when undergoing an inflammatory event. However, the Spz-eGFP KI reporter has been proven to be an insufficient imaging tool as no green fluorescent signal was detected in both transgenic and knock in

stage. The absence of a fluorescent signal in the reporter flies could be due to two possibilities: the GFP intensity is too low to be detected or a frameshift occurred in the plasmid during the multiple cloning processes. Various approaches were employed to examine the presence of GFP in the homozygous knock in reporter flies including live imaging using confocal and immunostaining with specific anti-GFP antibody. Nonetheless, none of the technique enabled us to detect the GFP signal in the Spz^{eGFP} homozygous knock-in reporter flies indicating the lack of fluorescent signal is not owing to its low intensity. Using quantitative PCR, the transcript of the green fluorescent protein was detected but in a significantly lower level in comparison to the transgenic Spz-neGFP reporter (Fig. 4.5B). Thus, the lack of GFP signal in the Spz^{eGFP} homozygous knock-in reporter might be due to a frameshift in the plasmid. Frameshift that occurred in the knock-in plasmid during the multi-steps cloning processes caused mismatch of the nucleotides, and that subsequently alters the protein sequence. Therefore, the transcript is present and could be detected by qPCR, but the GFP protein could be differ from the original mRNA. To confirm, the original pW25-Spz-eGFP KI construct will be sequenced.

Despite the lack of green fluorescence, the Spz^{eGFP} homozygous knock-in reporter has proven to be a useful *Spz* mutant in the study of the phenotypic consequences due to the absence of functioning Spz. It is the first and only known Spz null mutant with both endogenous loci replaced by green fluorescence protein, which was achieved by ends-out gene targeting (Huang et al., 2008). Functional analysis had been conducted to test the effect of the Spz null mutation on *Drosophila* survival in response to infection. Mutant and control flies were infected with bacteria (*M. luteus* and/or *E. coli*) or fungus (*C. albicans*). Our data shows that flies

become susceptible to fungal infection when the Toll pathway fails to be activated due to the lack of Spz. In contrast, bacterial infection with *M. luteus* and *E. coli* has a less dramatic impact on the survival of the Spz null mutant. Thus, *M. luteus* might not be a efficient Gram-positive bacteria to cause lethality but might be a good bacteria to study how the Toll pathway responses and clear the bacterial infection.

The Spz^{eGFP} knock-in reporter will be a useful tool, which allows us to extensively study functions of Spz in both developmental biology and immunology. To date, 31 Spz mutants are reported and majority of Spz research were conducted using these loss of function and gain of function mutants. Yet, majority of these mutants are not ideal for studying the fly immune responses as they are lethal and/or result in different levels of maternal effect. The most commonly used mutants are spz^{2}, spz^{3} and spz^{4}, in which the mutations were induced by mutagen ethyl methanesulfonate. Embryos produced by homozygous spz^{2} females are all dorsalised (Carroll et al., 1987) and thus this mutant is widely used in studying embryogenesis and developmental biology. spz^{3} is a temperature sensitive allele and homozygous *spz3* females also produce dorsalised embryos (Apidianakis et al., 2005). Out of the three loss of function mutants, spz^{4} is heavily used in immunity studies, including the important discovery of the antifungal role of Toll and Spz (Lemaitre et al., 1996). However, recessive mutation of this allele also displays maternal effect in embryonic cuticle at the dorsal side (Morisato and Anderson, 1994) and causes muscle defect in both embryos and larvae (Halfon and Keshishian, 1998). Comparing to existing loss of function Spz mutants, no maternal effects were observed in the Spz^{eGFP} null mutant as maternal mRNA laid by heterozygous is not affected. Embryonic development in the null mutant also

appears to be normal with no defect in any tissues detected. Therefore, this novel Spz-eGFP knock in (heterozygous) and knock out (homozygous) reporters are a better mutant to study Spz biology in both aspects of development and immune system.

In conclusion, a novel Spz-eGFP knock in reporter was generated primarily for the purpose of a *in vivo* genome wide screening in order to identify potential upstream regulators in the Toll pathway. Despite various approaches to detect GFP signals in this reporter, no fluorescence was detected and thus proven it as an insufficient imaging tool. However, the homozygous knock in reporter is a Spz null mutant that provides a complete deletion of Spz with no developmental defects. Although the Spz-eGFP knock in reporter is not beneficial for the genome wide screen that will be heavily base on imaging with confocal microscopy, this reporter will still be a useful tool to further investigate and confirm the regulatory role of any novel candidates identified in the screen.

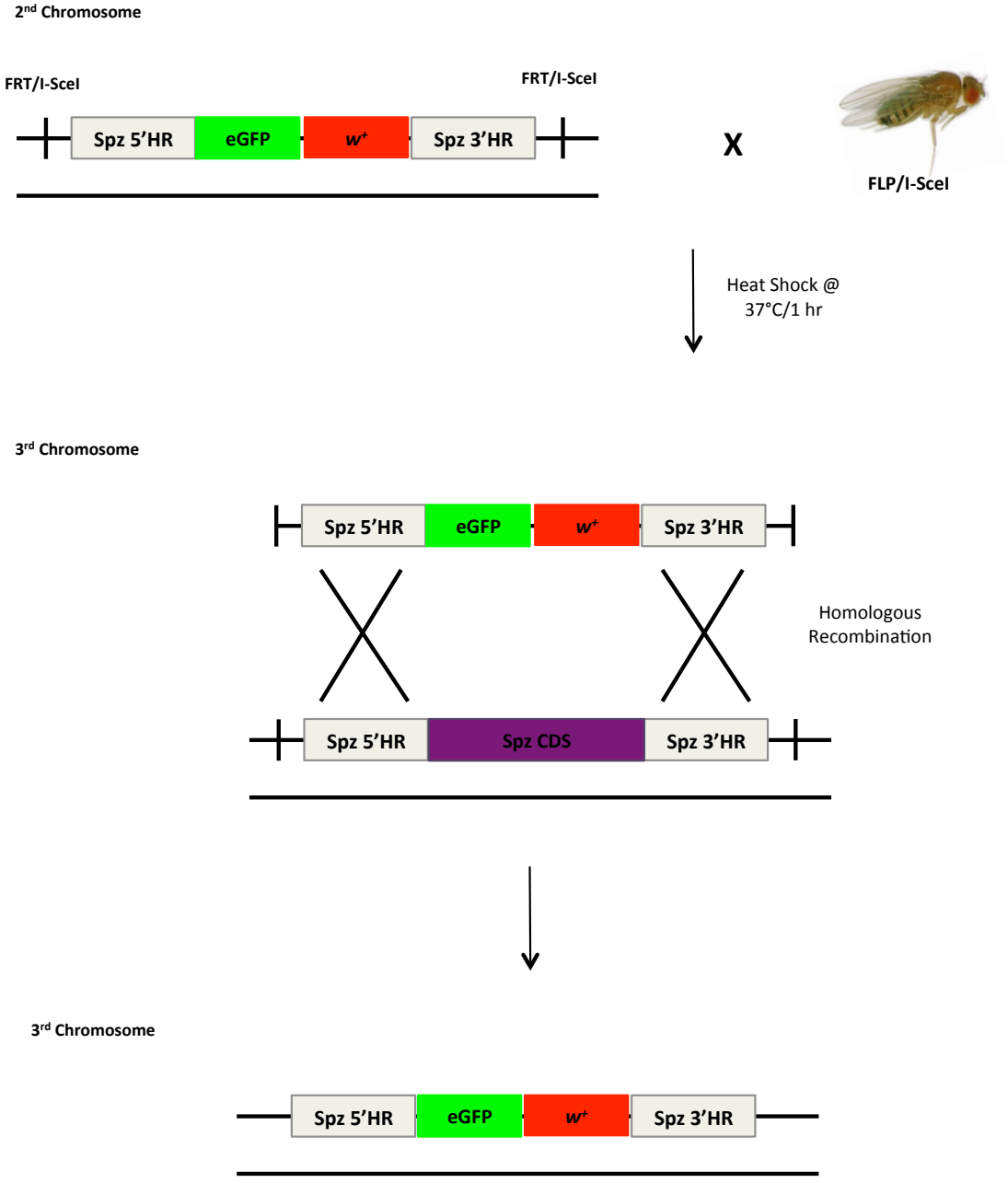


Figure 4-1: ***Schematic illustration of the knock in process through homologous recombination in Drosophila.*** Virgin females with the Spz-eGFP KI construct inserted into the 2nd chromosome were used to cross with the male FLP/I-SceI flies. The embryos were heat shocked at 37°C for 1 hour and the progenies were screened for their eye colour and wing phenotype, only virgin females with red/orange eyes and wild type wings would be picked out and crossed with a 3rd chromosome balancer line (w;;Tm2/Tm6c,Sb[1]). Through homologous recombination using the 3kb long 5' and 3' homology regions, the *Spz* CDS on the

3rd chromosome would be replaced by the eGFP and hence generated the KI reporter (Huang et al., 2008).

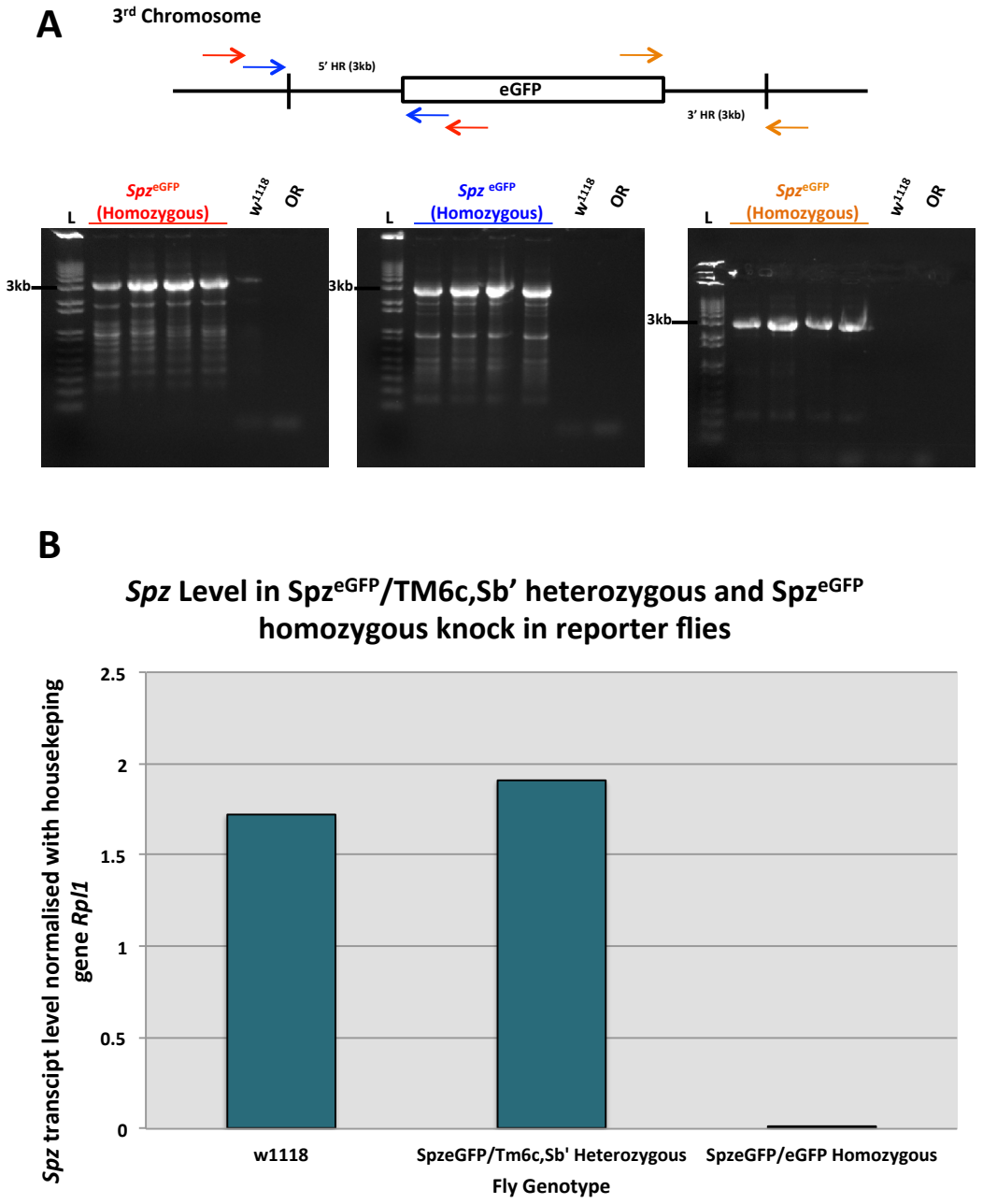


Figure 4-2: ***Genotyping and protein transcript level of Spz^{eGFP} KI flies.***

(A) Genomic DNA were extracted from Spz^{eGFP} homozygous knock in flies [Spz^{eGFP} (Homozygous)] and used as the template for genotyping with PCR. Various sets of PCR primers were designed overlapping the genomic DNA upstream or downstream of the knock-in site, the 5'HR or 3'HR, and the eGFP. Long range PCR was performed to confirm successful insertion of the knock in fragments. Chromosomal locations of the left and right primers are indicated by arrows and the corresponding agarose gel picture for each primer set are indicated by the

colour of the arrow. Red arrows indicate long range PCR performed using Primer set E and the PCR product is 3616bp long. Blue arrows indicate long range PCR with Primer set F and the produce a fragment with size of 3174bp. Orange arrows show the long range PCR Primer set G and the PCR product is 3106bp in size.

(B) The *Spz* transcript level of the Spz^{eGFP}/TM6c,Sb^{1} heterozygous (Spz^{eGFP}/TM6c,Sb^{1} Heterozygous) and Spz^{eGFP} homozygous reporter (Spz^{eGFP} Homozygous) was addressed by real time-PCR. The *Spz* level is normalised with housekeeping gene *Rpl*1. In Spz^{eGFP}/TM6c,Sb^{1} reporter, the *Spz* transcript level is with no significant compared to the wild type control w^{1118}. In the Spz^{eGFP} homozygous knock in flies, no *Spz* transcript was detected.

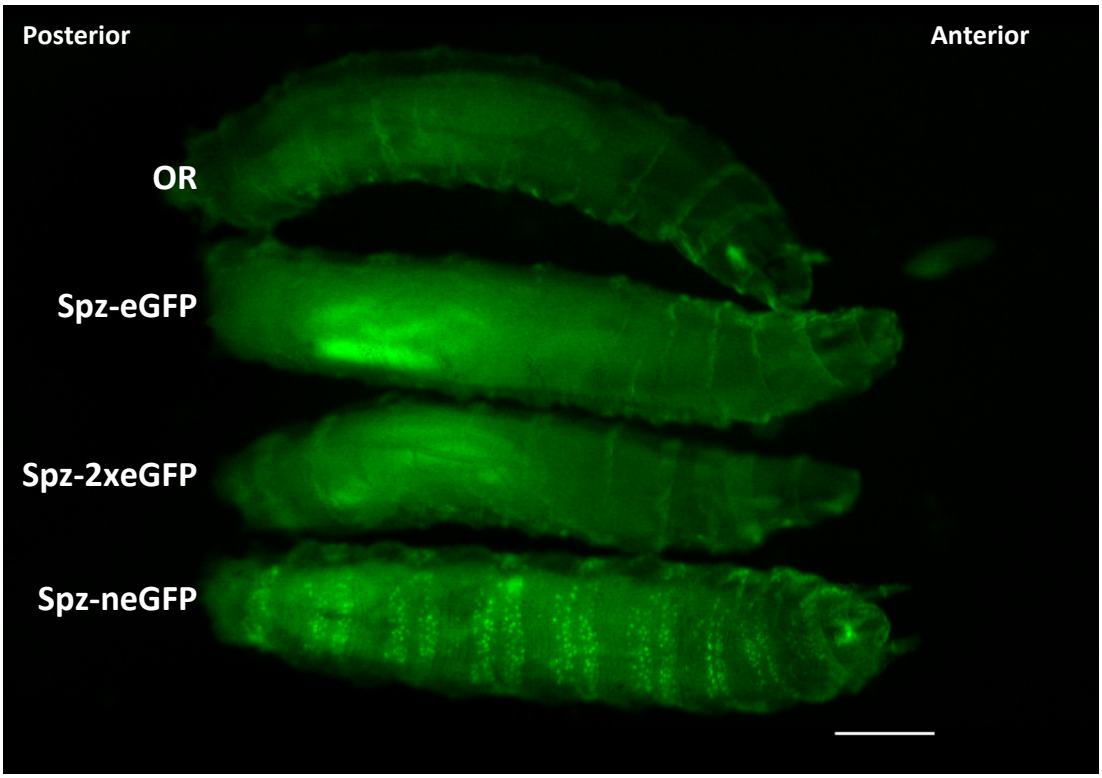


Figure 4-3: ***Observation of GFP signal in the transgenic 3rd instar larva with Spz-neGFP KI construct insertion.*** Wandering 3rd instar larvae of different genetic background were picked and imaged by fluorescent microscopy. The OR larva was used as the negative control for larvae with Spz-eGFP KI construct (Spz-eGFP), Spz-2xeGFP KI construct (Spz-2xeGFP), and Spz-neGFP KI construct (Spz-neGFP). GFP signals were only observed in the Spz-neGFP transgenic larva (Scale bar: 500μm).

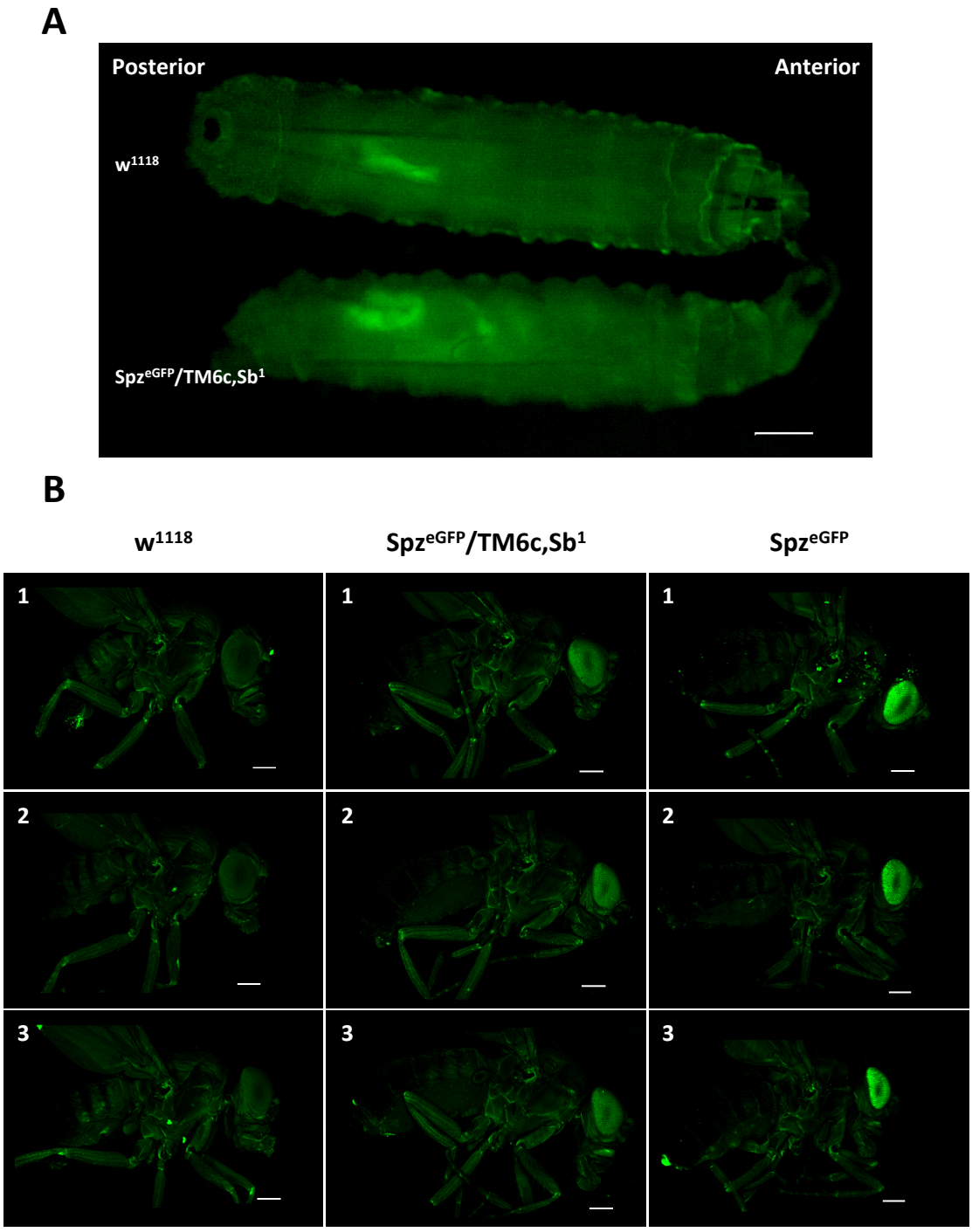


Figure 4-4: ***Observation of GFP signal of heterozygous Spz$^{eGFP/TM6c,Sb'}$ and homozygous Spz^{eGFP} knock in reporter by imaging at steady state.***

(A) No GFP signal was observed in the 3rd instar larvae of w^{118} and the $Spz^{eGFP}/TM6c,Sb^{1}$ homozygous knock in reporter flies ($Spz^{eGFP}/TM6c,Sb^{1}$) at steady state (Scale bar: 500μm).

(B) Age synchronised (4-7 days old) male adults of w^{1118}, Spz^{eGFP}/TM6c,Sb^{1} KI flies (Spz^{eGFP}/TM6c,Sb^{1}) and Spz^{eGFP} reporter flies (Spz^{eGFP}) were imaged by confocal microscope. No green fluorescence signal was observed in either heterozygous or homozygous Spz^{eGFP} knock in flies (Scale bar: 200μm).

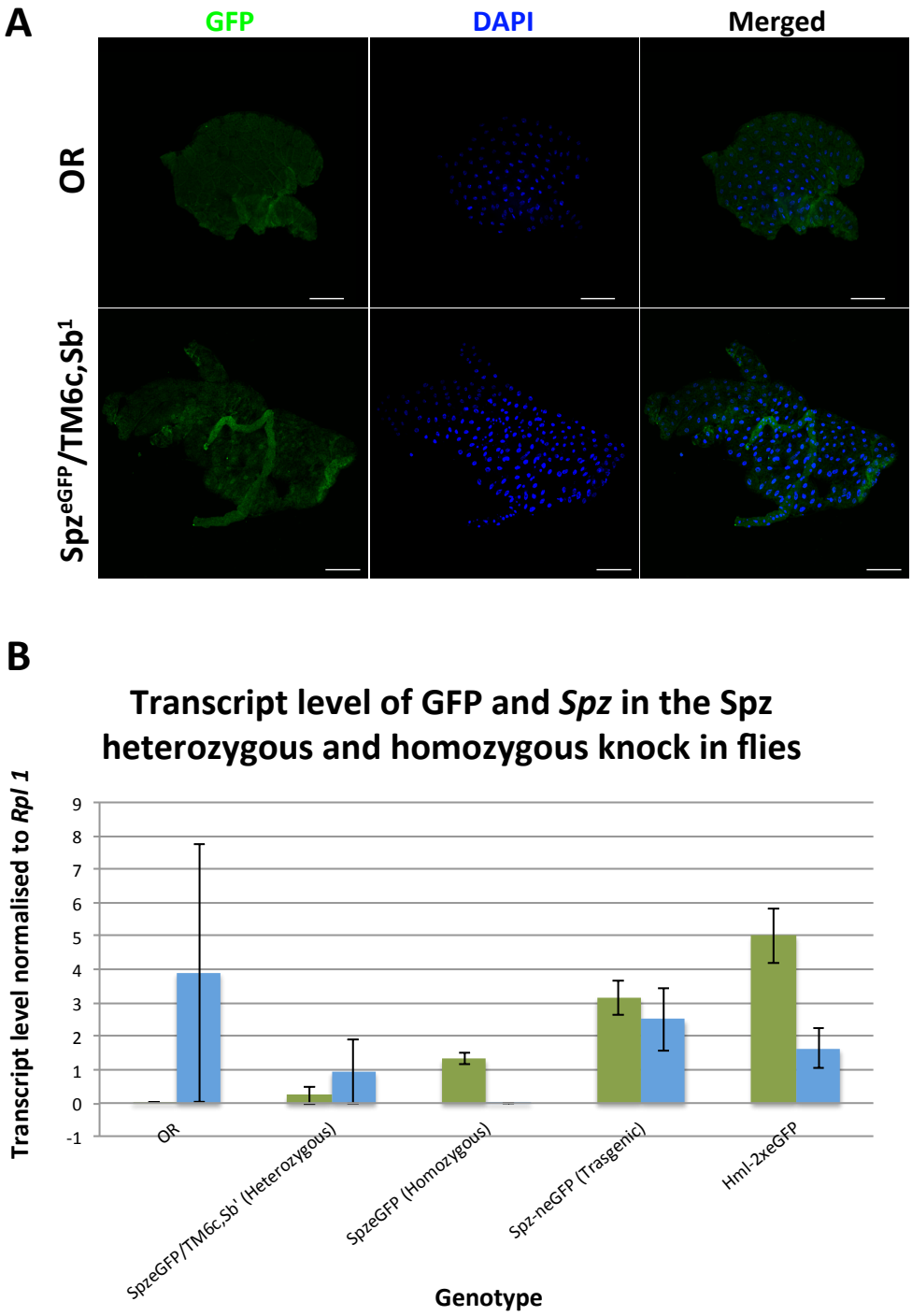


Figure 4-5: ***GFP is not produced in the Spz KI reporter flies confirmed by immunostaining and quantitative PCR.***

(A) Fat body tissues from wandering 3rd instar larvae of Spz^{eGFP}/TM6c,Sb^{1} KI flies were dissected for immunostaining with anti-GFP antibody. OR flies were used as

the negative control and are shown on the top panel. No GFP signal was detected (Scale bar=200μm).

(B) The transcript level of eGFP was quantified by quantitative PCR. Both eGFP and *Spz* level were normalised to the housekeeping gene *Rpl*1. GFP transcription was detected in both Spz^{eGFP}/TM6c,Sb^{1} heterozygous reporter [Spz^{eGFP}/TM6c,Sb^{1} (Heterozygous)] and Spz^{eGFP} homozygous mutants [Spz^{eGFP} (Homozygous)] but the expression was significantly lower than the Spz-neGFP transgenic reporter [Spz-neGFP (Transgenic)].

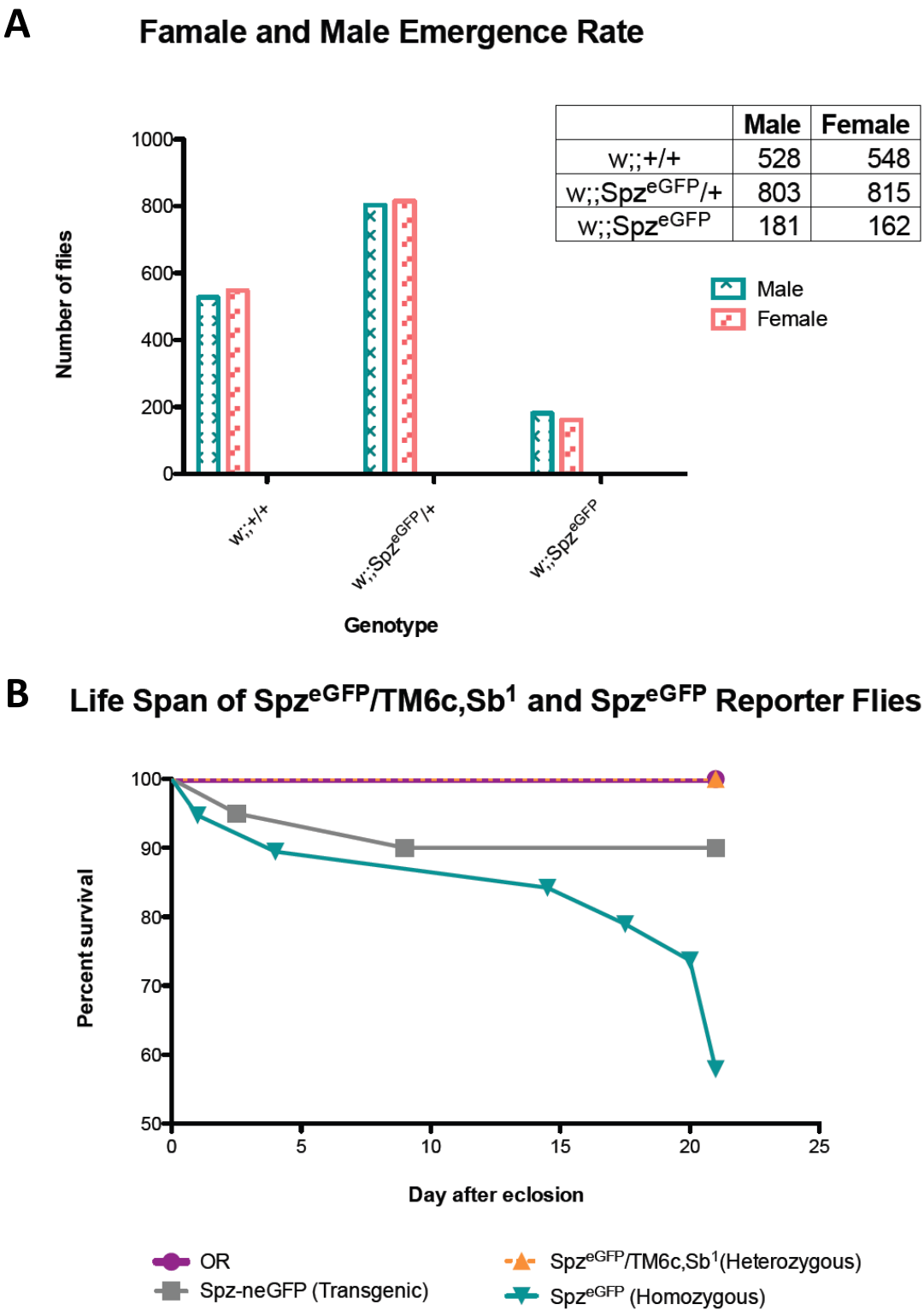


Figure 4-6: ***Phenotypic characterisation of the Spz^{eGFP}/TM6c,Sb^{1} and Spz^{eGFP} knock in flies.***

(A) No difference in female and male emergence rate was observed among w;;+/+, Spz^{eGFP}/+ heterozygous and Spz^{eGFP} homozygous knock in flies after back crossing with w^{1118}. Flies were collected and counted from 20 individual vials with total number of 3037 flies over a period of 30 days.

(B) Life span of the Spz-neGFP transgenic reporter [Spz-neGFP (Transgenic)], Spz^{eGFP}/TM6c,Sb^{1} heterozygous knock in flies [Spz^{eGFP}/TM6c,Sb^{1} (Heterozygous)] and Spz^{eGFP} homozygous knock in flies [Spz^{eGFP} (Homozygous)] had been measured. The Spz^{eGFP} homozygous knock in flies exhibited a reduced life span in normal condition. n=1, 20 flies per each genotype.

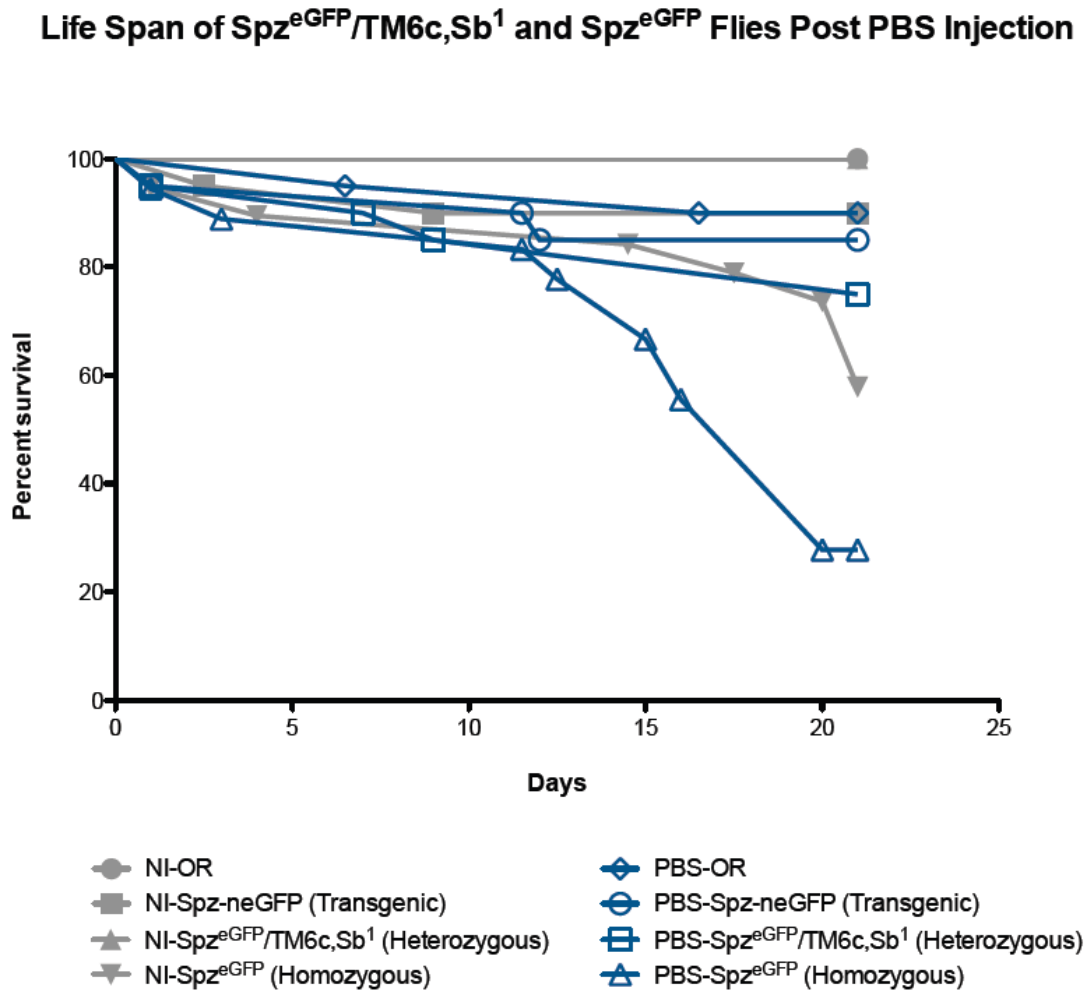


Figure 4-7: ***The Spz^{eGFP} homozygous knock in flies are sensitive to wounding.*** Age synchronised (4-7 days old) virgin female heterozygous and homozygous Spz^{eGFP} reporter flies injected with sterile PBS showed a declined survival. Only 28% of the PBS-injected homozygous knock in flies [Spz^{eGFP} (Homozygous)] were able to survive to 21 days post injection versus 78% for non-injected mutants, while 75% of the heterozygous Spz^{eGFP}/TM6c,Sb^{1} reporter flies [Spz^{eGFP}/TM6c,Sb^{1} (Heterozygous)] survived injection with sterile PBS. These data indicated that lack of *Spz* increases the susceptibility to injury and wounding. n=1, 20 flies per each genotype. The NI control shown in this figure is the same set as Fig. 4-6B. The NI control was performed at the same time as the PBS injection.

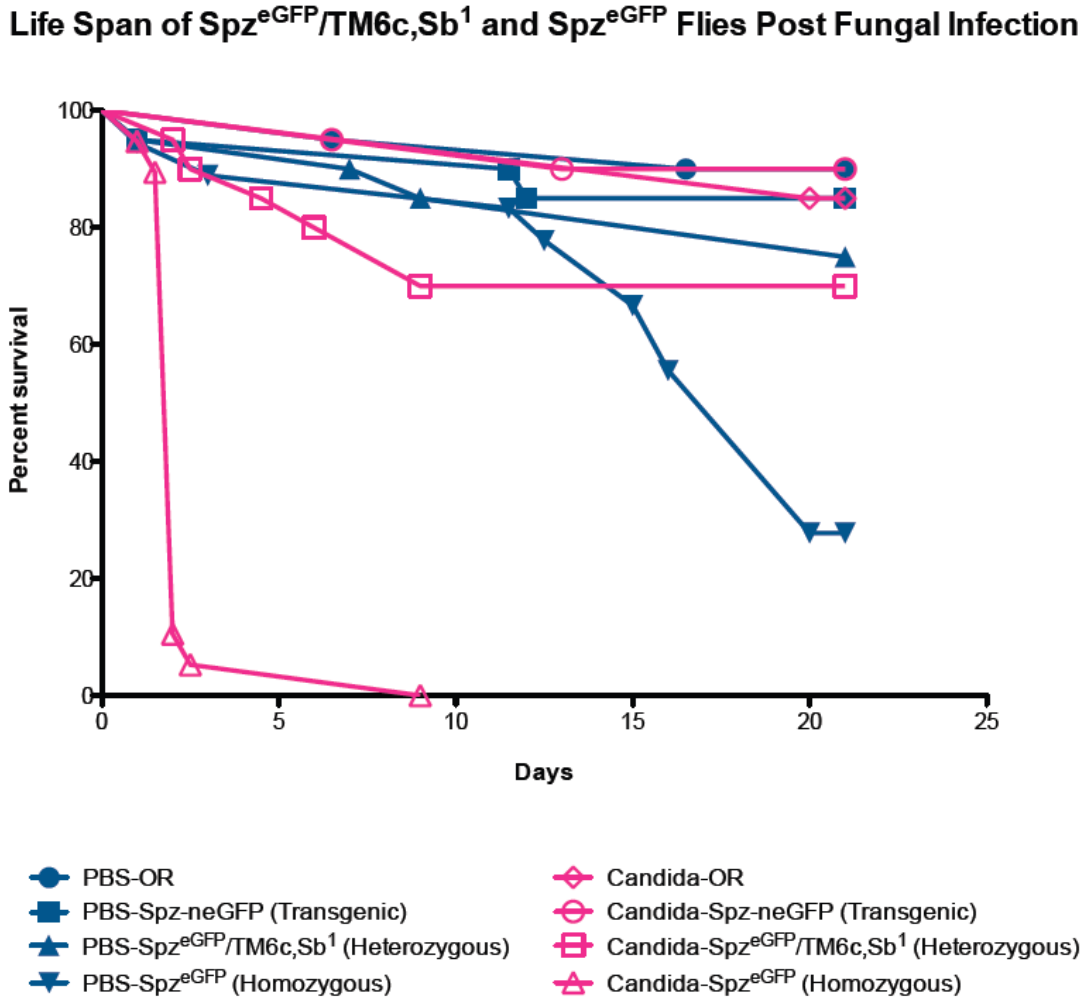


Figure 4-8: ***Fungal infection reduced the life span of Spz^{eGFP} homozygous knock in flies dramatically.*** Only 5% of the young (4-7 days old) virgin female homozygous Spz^{eGFP} reporter flies [Spz^{eGFP} (Homozygous)] were able to survive up to 2.5 days post infection with *C. albicans*. The heterozygous Spz^{eGFP}/TM6c,Sb^{1} knock in flies [Spz^{eGFP}/TM6c,Sb^{1} (Heterozygous)] exhibited a slight reduction in their resistance to fungal infection as 30% of the flies died in the first 10 days following fungal injection. n=1, 20 flies per each genotype. The NI control shown in this figure is the same set as Fig. 4-6B. The NI control was performed at the same time as the fungal infection.

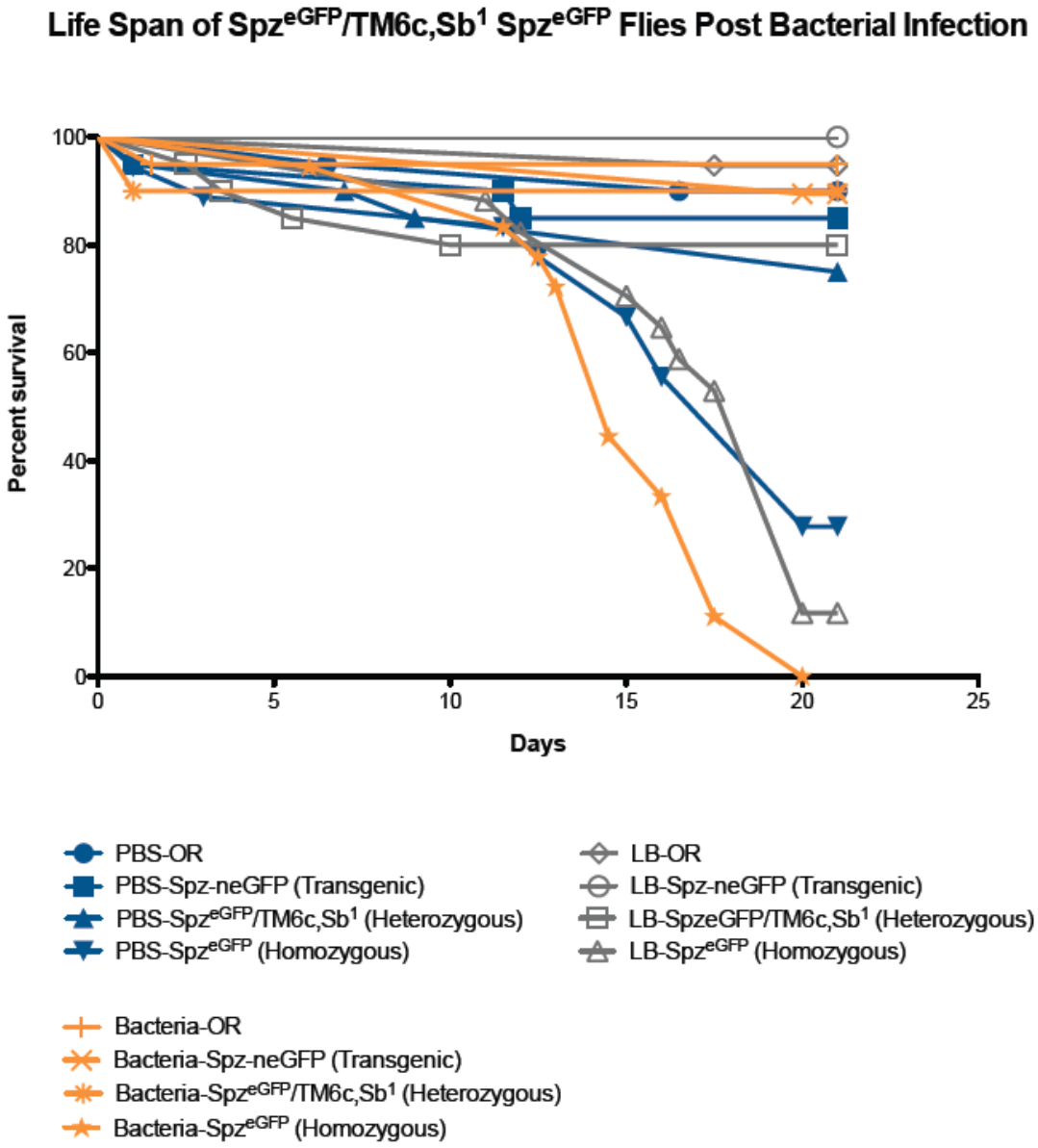


Figure 4-9: ***Homozygous Spz^{eGFP} reporter flies are sensitive to Gram-positive and Gram-negative bacterial infection.*** 4 to 7 days old homozygous and heterozygous knock in virgin females were injected with a bacteria mixture containing *E. coli* and *M. luteus*. Homozygous knock in flies [Spz^{eGFP} (Homozygous)] displayed a shorten life span compared to flies that were injected with sterile PBS. All homozygous reporter flies died by 20 days after bacterial infection whereas 27% and 11% of the reporter flies survived the injection with sterile PBS and LB Broth, respectively, on day 20. n=1, 20 flies per each genotype. The NI control shown in this figure is the same set as Fig. 4-6B. The NI control was performed at the same time as the PBS injection and bacterial injection.

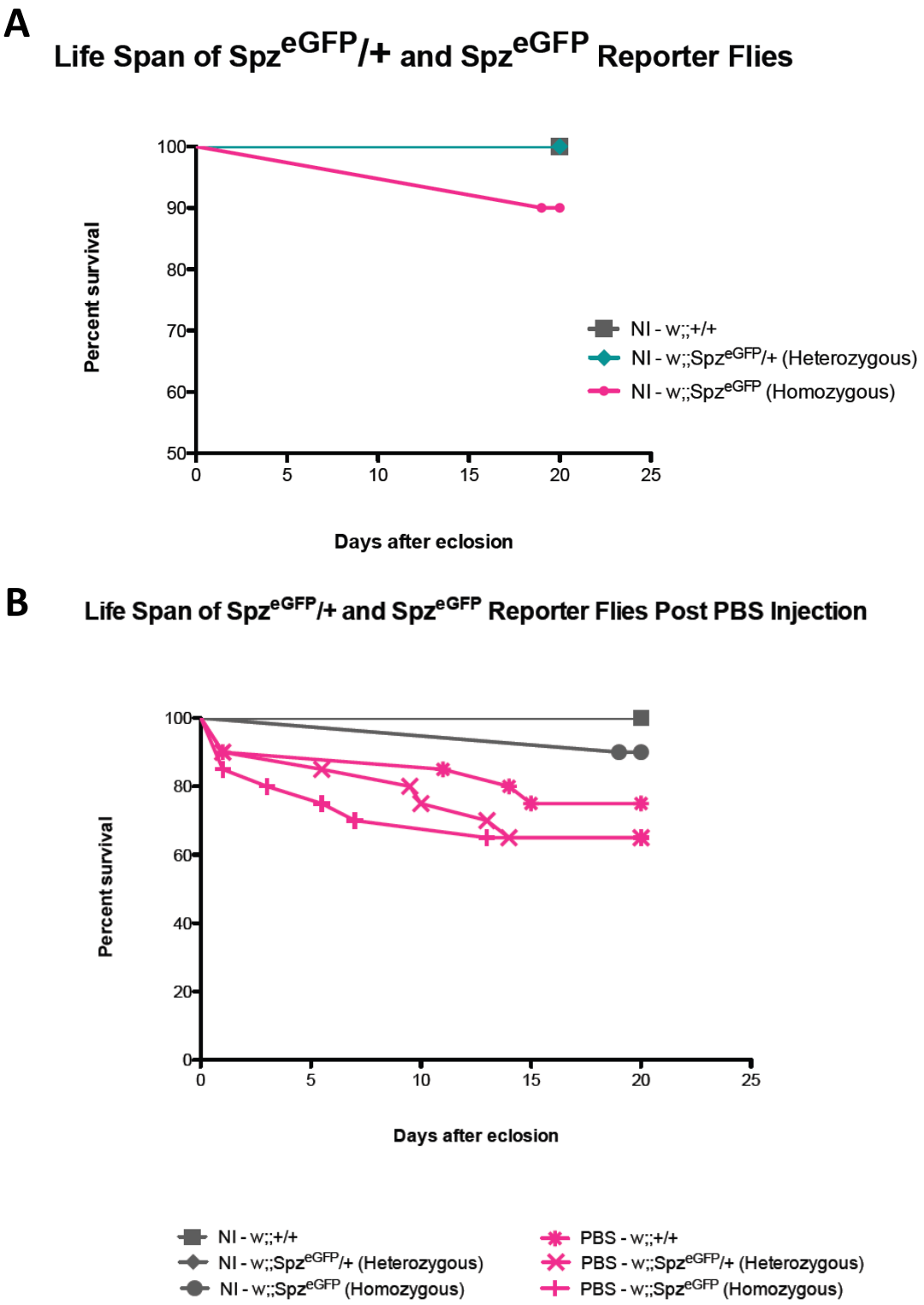


Figure 4-10: ***Spz^{eGFP} homozygous reporters obtained from the backcrossed stock displayed a prolonged life span and a higher survival rate upon injury.*** (A) Life span of age synchronised (4-7 days old) heterozygous and homozygous

knock in males during normal condition. 90% of the Spz^{eGFP} homozygous reporter flies [Spz^{eGFP} (Homozygous)] lived longer than 20 days whilst all w;;+/+ and heterozygous Spz^{eGFP}/+ reporter flies [Spz^{eGFP}/+ (Heterozygous)]were alive at day 20. n=1, 20 flies per each genotype.

(B) The homozygous Spz^{eGFP} reporters displayed a higher survival rate upon wounding due to injection with sterile PBS. 65% of the Spz^{eGFP} homozygous reporter obtained from the backcrossed stock survived 20 days post PBS injection whereas 28% of the homozygous Spz^{eGFP} knock in flies from the original balanced stock survived in the first 20 days after wounding. n=1, 20 flies per each genotype. The NI control shown in this figure is the same set as Fig. 10A.

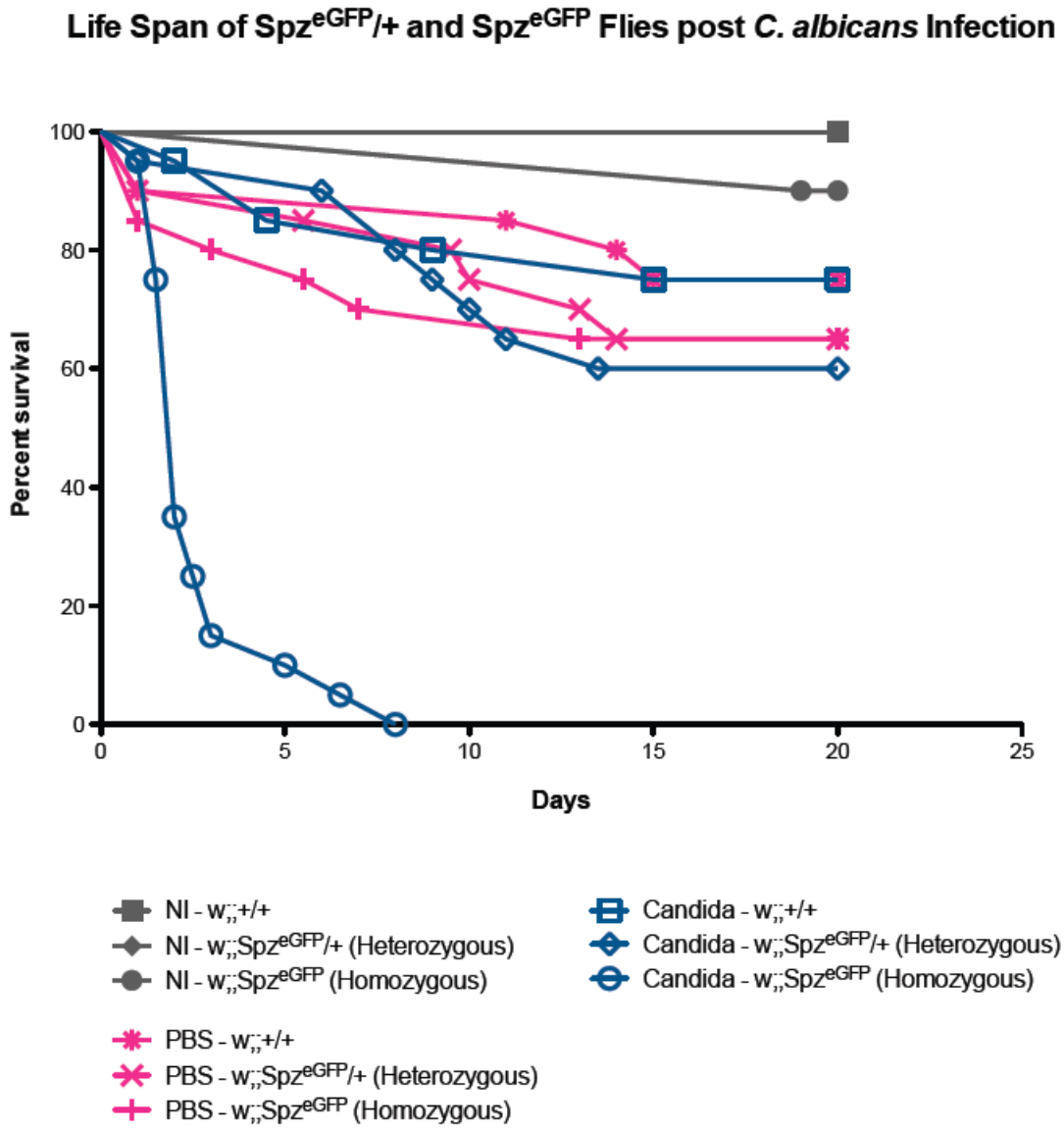


Figure 4-11: ***Homozygous Spz^{eGFP} reporters from the backcrossed stock exhibited a higher resistance to infection with fungus C. albicans.*** At 2.5 days post fungal infection, 25% of the Spz^{eGFP} reporters [Spz^{eGFP} (Homo)] survived when 95% of the Spz^{eGFP} reporters from the original balanced stock died at this time point. The Spz^{eGFP} reporters from the backcrossed stock reached the 95% death rate at 6.5 days after *C. albicans* infection but all the flies died at day 8 post infection, a day earlier then Spz^{eGFP} reporters from the original stock. The survival experiment was conducted using 4 to 7 days old male reporter flies. n=1, 20 flies per each genotype. The NI control and PBS injection shown in this figure is the same set as Fig. 4-10A and Fig. 4-10B.

- Chapter 5 -
Characterisation of a *Spätzle* Transgenic Reporter

5.1 Introduction

A new transgenic Spz reporter that is independent of the Gal4-UAS system was generated during synthesis of the Spz knock in (KI) reporter. A nucleus-eGFP (neGFP) is inserted downstream of the Spz coding region and thus driving the expression of neGFP at the location where Spz is expressed. Expression of Spz in the flies from mid embryogenesis (stage 11) until adult stage was characterised using this new transgenic Spz-neGFP reporter flies and confocal microscopy. It allows us to obtain insight of the expression time and pattern of the Spz during each stage of development and a good tool to focus on the dynamic of the cytokine while undergoing immune challenge.

5.2 Spz expression during different developmental stages

Spz is important both during embryogenesis and immune responses against pathogens (Morisato and Anderson, 1995; Valanne et al., 2011). It is known that Spz has an important contribution to the Dorsal-Ventral patterning during early stages of embryogenesis. Maternal Spz mRNA is placed in the embryos inside the ovaries during oogenesis and when the egg is fertilised and laid, the mRNA is transcribed to trigger activation of the Toll signalling pathway leading to activation of the Dorsal protein that defines the dorsal-ventral axis bases on the concentration of Dorsal. However, it is not known when embryonic *Spz* is being transcribed, the contribution of *Spz* during development and a possible role during infection and other immune condition such as wounding. By characterising the novel Spz-neGFP transgenic reporter, it allows us to observe *Spz* transcription in

different tissues revealed by GFP throughout embryogenesis using fluorescent or confocal microscopy.

This novel reporter was generated in the process of making the Spz KI reporter. Out of the three KI constructs - Spz-eGFP, Spz-2xeGFP, and Spz-neGFP - only the Spz-neGFP KI construct showed green fluorescent signal in 3rd instar larvae at the pre-knock-in transgenic state and hence was selected for characterisation to confirm that it is a faithful reporter for *Spz* (shown in Fig. 4-3). The Spz-neGFP KI construct in the pW25 plasmid contains two 3kb long homology regions from upstream and downstream of the *Spz* gene and a nuclear eGFP (neGFP) protein (shown in Fig. 3-3D). The construct was inserted randomly into the genome. To characterise the Spz-neGFP transgenic reporter, embryonic, larval, and adult *Drosophila* were examined and *Spz* expression revealed by GFP were examined during steady state. The characterisation will provide us an insight into the kinetics and locations of *Spz* expression in *Drosophila* throughout its lifespan.

5.3 Expression pattern of the Spz-neGFP transgene in various stages of Drosophila embryos

Different stages of *Drosophila* embryos were collected and mounted in low-melt agarose for intravital imaging. No GFP signals were detected in the early stages of embryogenesis and only from embryonic stage 11 onwards was GFP fluorescence observed. Between embryonic stages 11 to 13, GFP fluorescence was detected in the putative embryonic epidermal cells. They lasted until the embryo emerged as a 1st instar larva (Fig. 5-1, Top 2 panel). At stage 14, GFP expression was detected in

embryonic hemocytes (Fig. 5-1, Middle two panels). During embryonic haematopoiesis, plasmatocytes begin to migrate out of the head when germ band retraction starts at stage 12 and by stage 14 plasmatocytes are evenly distributed in the embryo. Embryonic plasmatocytes populate the embryo via four distinct routes: 1). mid-ventrally between the ventral epidermis and the ventral cord; 2). between the dorsal surface of the ventral nerve cord and the mesoderm; 3). along the dorsal boundary of the epidermal primordium; and 4). along the gut primordium (Tepass et al., 1994). With an appropriate fluorescent hemocyte reporter, three of the migratory routes can be observed on the ventral surface of the embryo (Fig. 5-2A, Bottom panel). By mounting a stage 14 embryo of the transgenic reporter on its ventral side, GFP signals were observed in cells along the three migration routes (Fig. 5-2A, Top panel). To determine the cell type of these GFP positive cells, the Spz-neGFP transgenic reporter was crossed with a hemocyte reporter, *Singed-Ga4* (Sn-Gal4), where embryonic hemocytes are labelled by a nucleus RFP (w;Sn-Gal4,UAS-RedStinger) (Barolo et al., 2004). Sn-Gal4 was first described as the *Drosophila* gene encoding a protein that is homologous to the sea urchin and vertebrate fascin, an actin-bundling protein that has a fundamental contribution in actin organisation during oogenesis and bristle extension (Bryan et al., 1993; Tilney et al., 2000). It had later been discovered to play a significant role in the motility of migrating cells in the fly embryo (Zanet et al., 2009a). Fascin is highly expressed in *Drosophila* hemocytes during embryonic development and is important for hemocyte migration for both embryogenesis and inflammatory responses induced by epithelial wounding (Zanet et al., 2009a; Zanet et al., 2009b). Using the Sn-Gal4 reporter, hemocytes in embryos were observed as early as in stage 11 to 12 using confocal microscopy (Supplementary Fig. 1).

With *in-vivo* imaging, co-expression of GFP and RFP in these cells was discerned, thus confirming that the GFP positive cells populating the embryo body at this stage were embryonic hemocytes (Fig. 5-2B). In addition to hemocytes, localised expression of green fluorescence was also detected in the developing salivary glands and two group of cells located at the anterior and posterior end of the embryo (Fig. 5-2A). GFP expression persisted in embryonic hemocytes until late stage 17 and could be observed in migrating blood cells found in the anterior and posterior regions (Fig. 5-3; Movie 1). Additionally, at late stage 17, the salivary glands were also GFP positive together with the mouth hook and the hindgut located at the posterior end of the embryo (Fig. 5-1, Bottom panel). Therefore, we believed that the two localised groups of cells with GFP expression found at stage 14 at both ends of the embryo could be the anlage of the mouth hook and the gut rudiment, respectively.

5.4 Expression of the Spz-neGFP reporter in 3rd instar larvae

To study Spz expression in the larval life stage, 3rd instar larvae were examined using a fluorescent microscope (Leica). The epidermis of the 3rd instar larvae showed GFP expression with horizontal patterns located at the interval of each cuticle segments (Fig. 5-4). We were unable to determine the cell type of these GFP positive cells due to the lack of a suitable larval epidermal reporter. Despite its transparent body, it was difficult to distinguish individual internal organs without opening the larvae. Hence, wandering 3rd instar larvae were dissected and fixed immediately *in-situ* (Fig. 5-5). The larval tissues were then labelled with anti-GFP

antibody to reveal GFP expression in specific organs. We have first looked at fat body tissues as *Spz* mRNA is constitutively expressed by the fat body even during non-challenged status (Irving et al., 2005). In healthy 3rd instar larvae, the Spz-neGFP transgenic reporter gene was found to be expressed by all fat body tissues (Fig. 5-6, Top panel). All salivary gland cells also displayed green fluorescence in unstimulated larvae (Fig. 5-6, Middle panel), but it is not clear whether *Spz* expression in the salivary glands reflects any immune functions. In the larval respiratory system, GFP signal was not detected in the two major tracheas but was detected in the epithelial cells of the small bunching tracheas (Fig. 5-6, Bottom panel). We have also examined the digestive system of the larva. Whole intestine was dissected and the hindgut was GFP positive (Fig. 5-7). The expression pattern in the hindgut suggests that the reporter gene could possibly be expressed by the intestinal epithelial cells (Fig. 5-7B). Interestingly, no fluorescent signals were detected in the midgut, which is a main site of immune responses (Buchon et al., 2010). Upon infection and immune challenge, damage to larval intestinal cells causes activation of different signalling pathways and production of antimicrobial peptides. These signalling pathways include the Imd pathway, the JAK/STAT signalling cascade and the EGFR pathway whilst Toll activity was undetectable (Buchon et al., 2010; Buchon et al., 2009). Following bacterial infection in the gut, stress induced stem cell proliferation as a consequence of the activation of the EGFR pathway replaces the damaged intestinal cells and hence remodels the gut (Buchon et al., 2010). It is thus surprising to reveal expression of Spz-neGFP transgene in the hindgut and the potential impact of *Spz* expression in this region in *Drosophila* gut immunity remains to be determined.

5.5 Expression pattern of the Spz-neGFP transgenic reporter in adult

All adult flies carrying the transgenic reporter gene displayed a bright fluorescence at the dorsal side of the head from the day of hatching (Fig. 5-8A). The ocellus showed no GFP expression but the signal around this area resembled a pattern or a network (Fig. 5-8A). We have compared the shape of the fluorescent structure and it exhibits similarity to the fat body tissues located in the head. We have also observed that the fluorescence intensity gradually declined while the fly was aging. Strongest GFP signals were detected on the day of hatching and all signal disappeared by 4 days after eclosion (Fig. 5-8B). Disappearance of fluorescence signals could be explained by two possibilities. As the adult fly develops, the body becomes less transparent and accompanied with enhanced autofluorescence due to increased pigmentations and thickened cuticle. The dorsal side of the head region of age synchronised transgenic reporter flies and wild type OR flies were examined. The complex eyes and the head region became more autofluorescent suggesting an increase in number of pigments and thickening of the cuticles (Fig. 5-8B). Nonetheless, the GFP signals in the Spz-neGFP transgenic reporter flies could still be detected by day 3 after birth suggesting the loss of green signal could not be attributed by pigmentation and thickened cuticles. Following emergence into adult fly, residues of larval fat body located in the head will gradually degenerate and become totally lost by the third day after hatching (Aguila et al. 2007). The loss of GFP signals in the reporter flies showed striking similarity to the degeneration time line of larval fat body tissues. Besides, the network shape observed around the ocellar triangle also resembles the structure of fat body

tissues. These observations thus indicate that these cells could be the remaining larval fat body cells. To determine whether *Spz* is also constitutively expressed by the fat body like in larvae, 10 days old adult males were picked and dissected. Due to the more fluid structure of adult fat body, the whole fly was dipped in 4% formaldehyde overnight before the tissues were carefully removed from the inner surface of the cuticle and labelled with anti-GFP antibody. The Spz-neGFP reporter genes were expressed by adult fat body tissues (Fig. 5-9). The crop and the digestive tract had also been isolated for immunostaining and both the crop (Fig. 5-10B) and the hindgut (Fig. 5-11B) remained GFP positive.

5.6 Induction of the Spz-neGFP reporter gene expression in adult hemocytes following exposure to bacterial agents

To show that this novel Spz-neGFP transgenic reporter is a useful reporter to monitor *in-vivo Spz* activity following immune challenge with specific attention to hemocytes, we have first crossed the Spz-neGFP transgenic reporter flies with a hemocyte reporter *Croquemort-Gal4* (Crq-Gal4) (Franc et al., 1996) (w;;Crq-Gal4, UAS-CD8-mCherry/TM6c,Sb^1, Supplementary Fig. 2) to generate the Hemocyte Transcriptional Reporter flies (Crosses as shown in Material and Methods 2.1.2.2) (Fig. 5-12) and infected 10 days old adult males with a bacteria mixture containing *M. luteus* and *E. coli* (Lemaitre et al., 1996). The bacteria mixture was injected into the flies (Fig. 5-13A) and the animals were allowed to recover from the injection at 25°C for 6 hours in a normal fly vial before intravital imaging with confocal microscope. During imaging, individual flies were immobilised on a cover slip on their backs with super glue and anesthetised with CO_2 (Fig. 5-13B). From our

preliminary results, induction of the Spz-neGFP transgene expression was observed in hemocytes located in the heart region (Fig. 5-13C). In the uninfected control, GFP fluorescence was detected in cells located on the back of the abdomen that were not hemocytes. Small number of hemocytes clustering around the heart area displayed expression of the Spz-neGFP reporter in the absence of immune challenge (Fig. 5-13C, Uninfected Control). Six hours after injection, expression of the transgenic reporter in these hemocytes have been upregulated (Fig. 5-13C, Infected 6hrs – Fly 1 to Fly 3). No difference in GFP expression in non hemocytic cells were observed but it might due to the fact that GFP appeared to be constitutively expressed by these cells and thus it is difficult to distinguish minor increase in fluorescence intensity before and after infection.

5.7 Discussions and conclusion

Following imaging of the novel Spz-neGFP transgenic reporter using confocal microscopy at various development stages, an expression profile of Spz is established. During embryonic development, Spz is expressed by the putative epidermal cells as earlier stage 11 and persist until 3rd instar larvae. From stage 14 to stage 17, embryonic hemocytes express Spz but we were unable to detect Spz expression in larval hemocytes. Spz expression was also observed in both larval and adult hemocytes without presence of immune challenge. Furthermore, constitutive expression of Spz was detected in the hindgut, possibly by the intestinal epithelial cells, in both 3rd instar larvae and adult. Spz is upregulated in hemocytes around the heart region following infection with a bacteria mixture containing *E. coli* and *M. luteus*.

Comparing to the latest available expression profile by sequencing tissue Spz mRNA on the online data base Flybase, it has shown that Spz are expressed in larval/adult hindgut; larval/adult carcass; larval fat body , trachea and salivary gland; the adult head and the adult female reproductive system. In addition, an expression profile in embryo has also been established by the Berkeley Drosophila Genome Project and it shows Spz expression by embryonic foregut and hindgut from stage 13. The expression profile of the Spz-neGFP transgenic reporter is comparable to the available database and thus suggesting this transgenic reporter is a faithful visual tool to study Spz.

This novel transgenic Spz-neGFP reporter will be a useful tool not only to study the kinetic of the cytokine during different stages of development and health status, it will also be a useful replacement for the *in vivo* genome wide screening to further investigate the transcriptional regulation of Spz. The original plan was to conduct the screen using a Spz^{eGFP} KI reporter. However, due to an uncertain reason (further discussed in Chapter 4, 4.4), no GFP signal was detected in the knock-in animal. The GFP signal in the transgenic reporter is strong enough for imaging purpose. Also, expression of the Spz-neGFP reporter gene is directly driven by the upstream and downstream regulatory segments of the Spz gene instead of the traditional Gal4-UAS system. Therefore, it will still allow us to conduct the genome wide RNAi screening using the Crq-Gal4 hemocyte reporter and ensure expression of the knock down effect remains hemocyte specific.

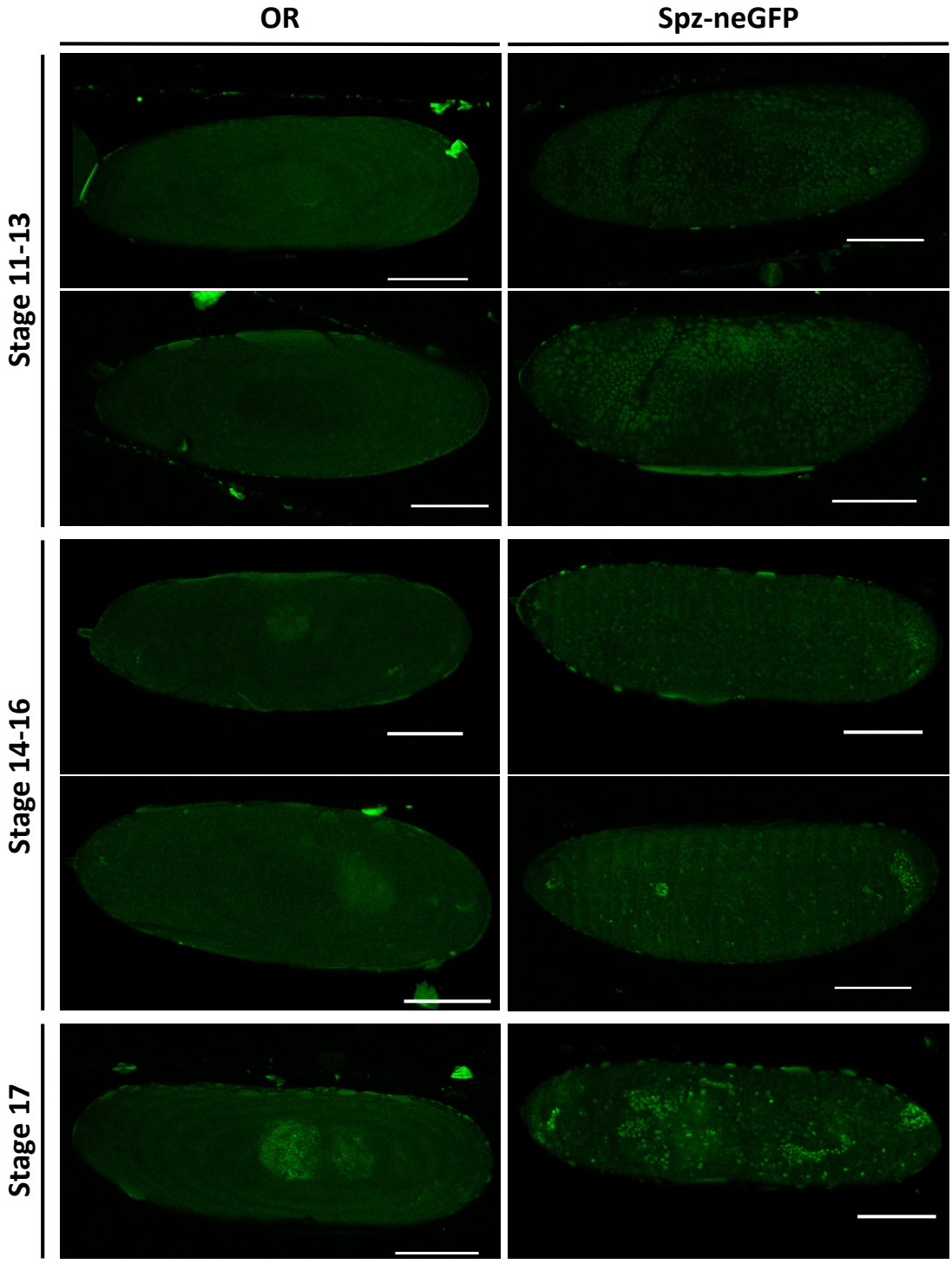
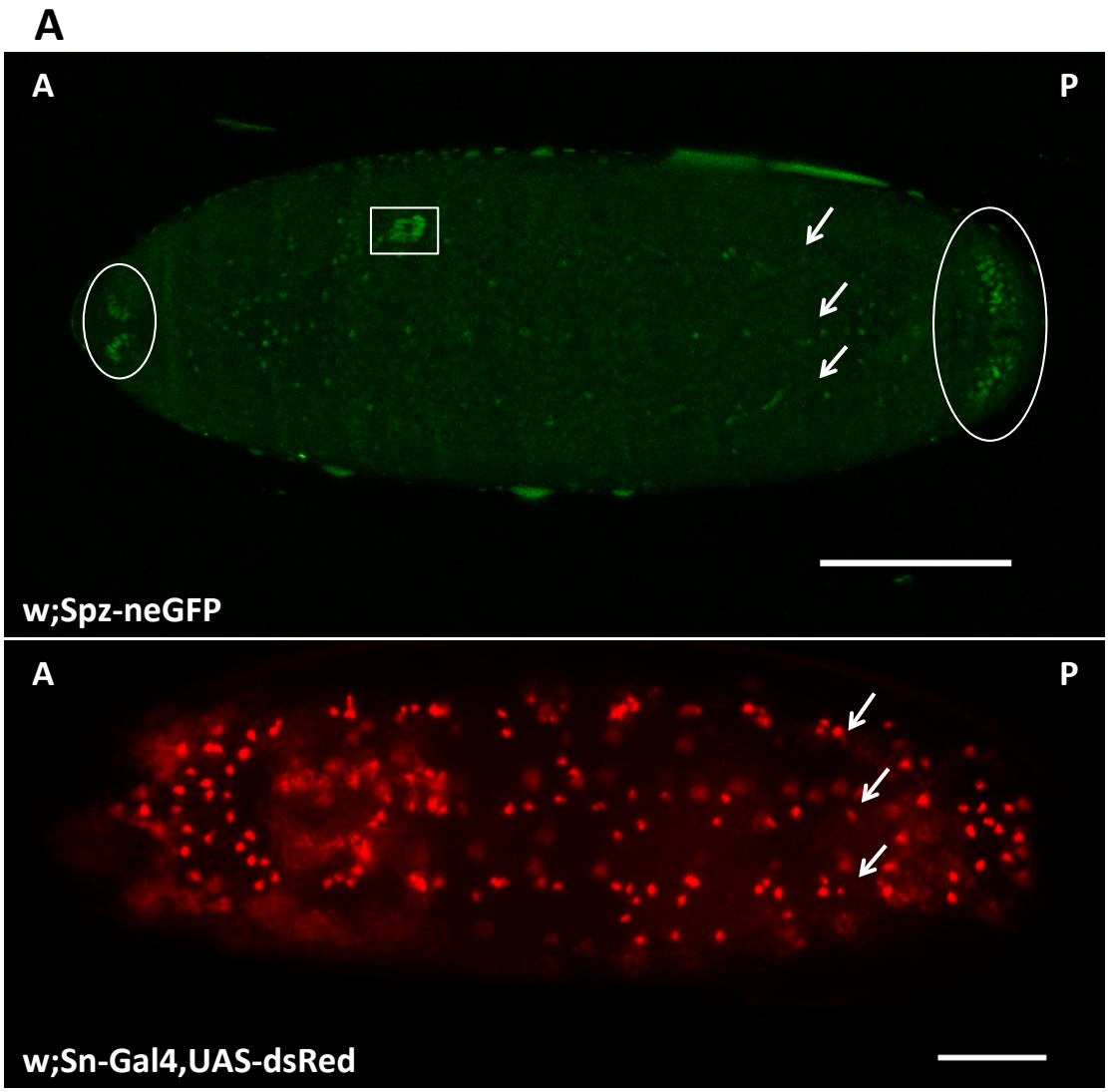


Figure 5-1: ***Expression pattern of the Spz-neGFP transgenic reporter during different stages of embryonic development.*** Age matched embryos of OR and Spz-neGFP transgenic reporter were collected, prepared and mounted in low melt agarose before *in-vivo* imaging. GFP expression in the embryos was not detected until embryonic stage 11 at the putative epidermal cells. From embryonic stage 14

onwards, embryonic hemocytes shown to be expressing the Spz-neGFP reporter. At embryonic stage 17, the salivary glands, mouth hook and the hindgut displayed green fluorescence.


A
A
P
w;Spz-neGFP
A
P
w;Sn-Gal4,UAS-dsRed

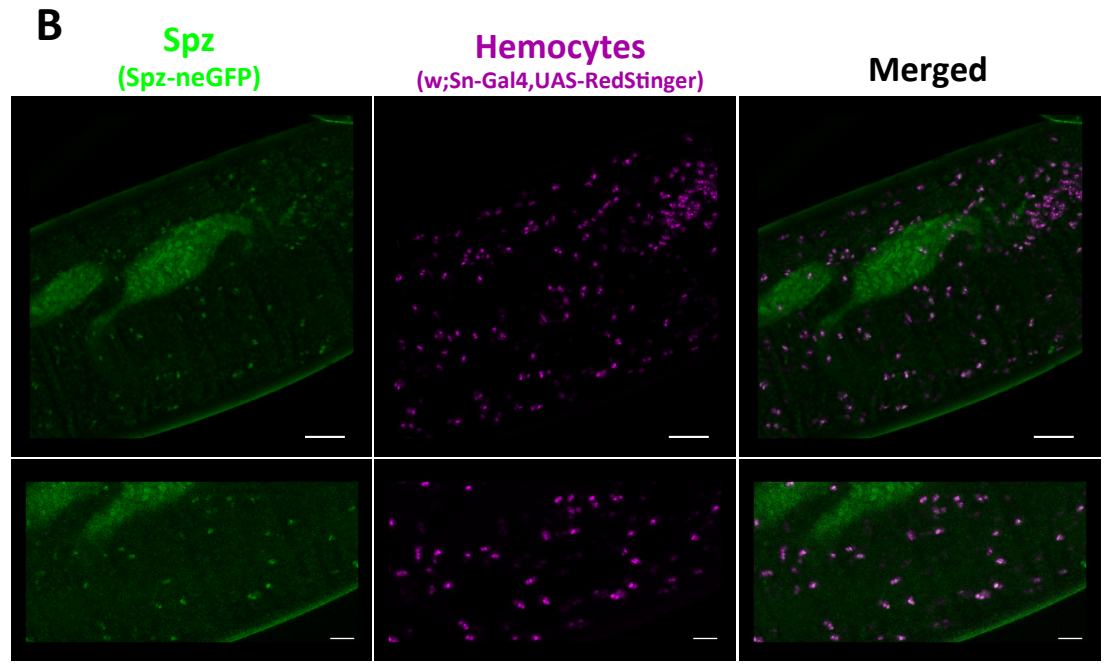

B
Spz
(Spz-neGFP)
Hemocytes
(w;Sn-Gal4,UAS-RedStinger)
Merged

Figure 5-2: ***Expression of the Spz-neGFP transgenic reporter in embryonic hemocytes from embryonic stage 14.***

(A) A stage 14 embryo of Spz-neGFP transgenic reporter was mounted on its ventral side. GFP were expressed by cells along the three hemocytes migratory routes (indicated by white arrow) and by the developing lymph gland (white square). Localised expression of GFP signal has also been observed in the mouth hook (white circle at A side) and the hindgut primordium (white circle at P side) at the anterior and posterior end of the embryo, respectively (A=Anterior; P=Posterior; Scale bar: 100μm). The lower panel shows a stage 13 embryo of w;Sn-Gal4,UAS-dsRed mounted on its ventral side. The three embryonic hemocyte migration routes are indicated by white arrows (A=Anterior; P=Posterior; Scale bar: 50μm).

(B) Crosses of Spz-neGFP with hemocyte reporter were made to confirm the GFP positive cells found along the migration routes on the ventral side of the embryo are embryonic hemocytes. Co-expression of GFP and RFP (false colour to be magenta) was detected in these cells in stage 15-16 embryo and hence confirming the Spz-neGFP reporter gene was exhibited by embryonic hemocytes (Scale bar - Top panel: 30μm; Bottom panel: 15μm).

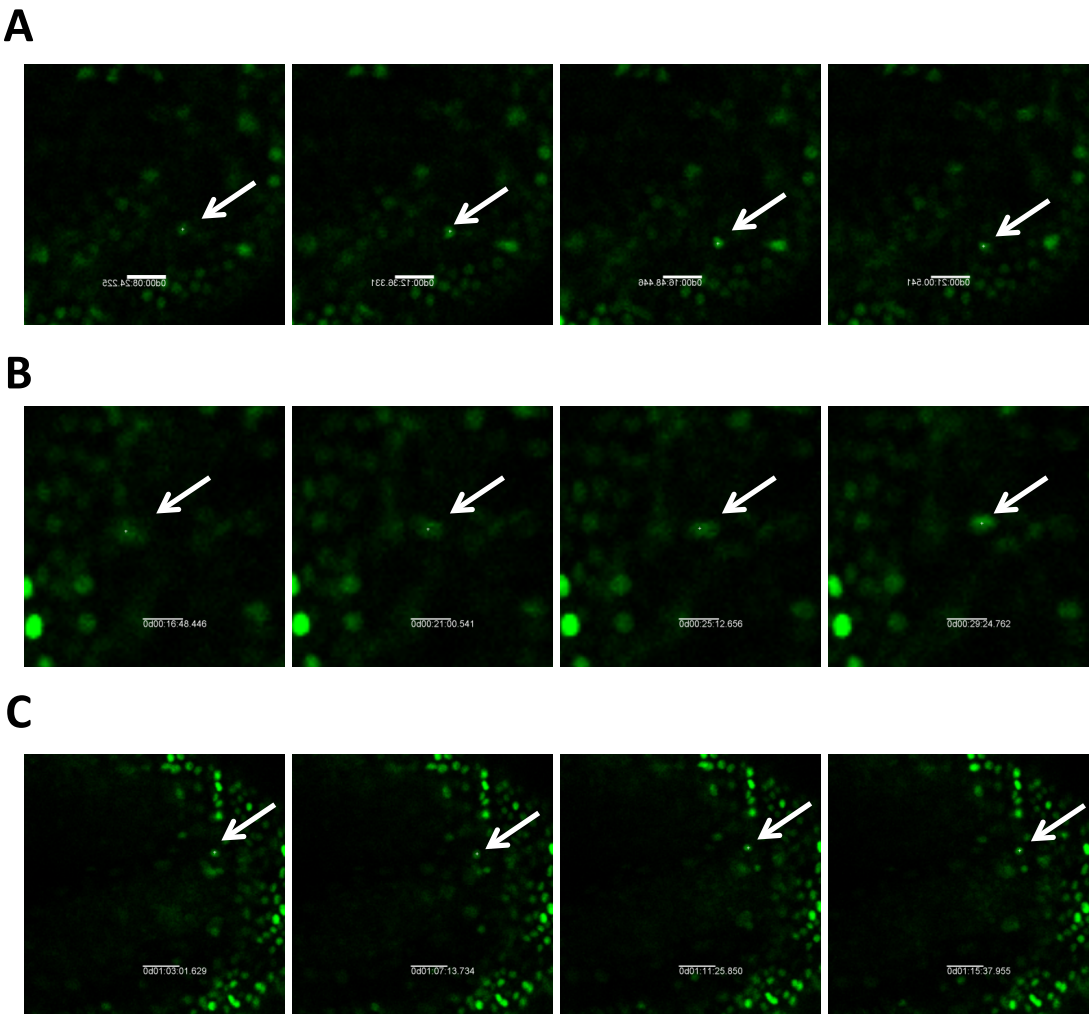


Figure 5-3: ***Expression of the Spz-neGFP transgenic reporter gene in migrating hemocytes in the anterior and posterior end of stage 17 embryo.*** (A) Time lapse imaging of migrating embryonic hemocytes (cell 1, pointed by write arrow) at the head region (Scale bar: 10μm).

(B) Time lapse imaging of migrating embryonic hemocytes (cell 2, pointed by write arrow) at the head region (Scale bar: 10μm).

(C) Time lapse imaging of migrating embryonic hemocytes at the rare end of the embryo (Scale bar: 10μm).

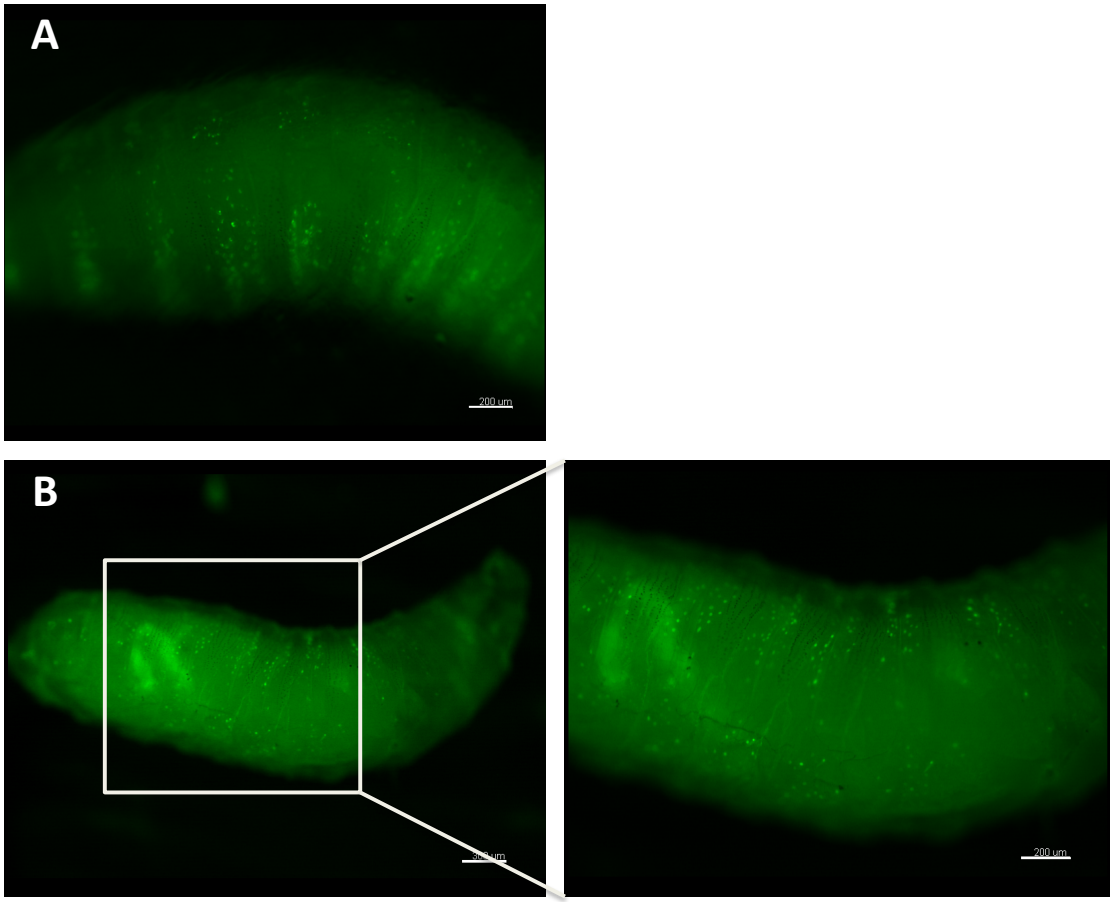


Figure 5-4: ***Expression pattern of Spz-neGFP reporter gene in the epidermis of 3rd instar larvae during steady state.*** The two larvae showed in (A) and (B) are two individual 3rd instar larvae picked and immobilised with ice-cold PBS during imaging with fluorescent microscope. Green fluorescence signal was displayed by epidermal cells situated in between each cuticle segments (Scale bar: 200μm).

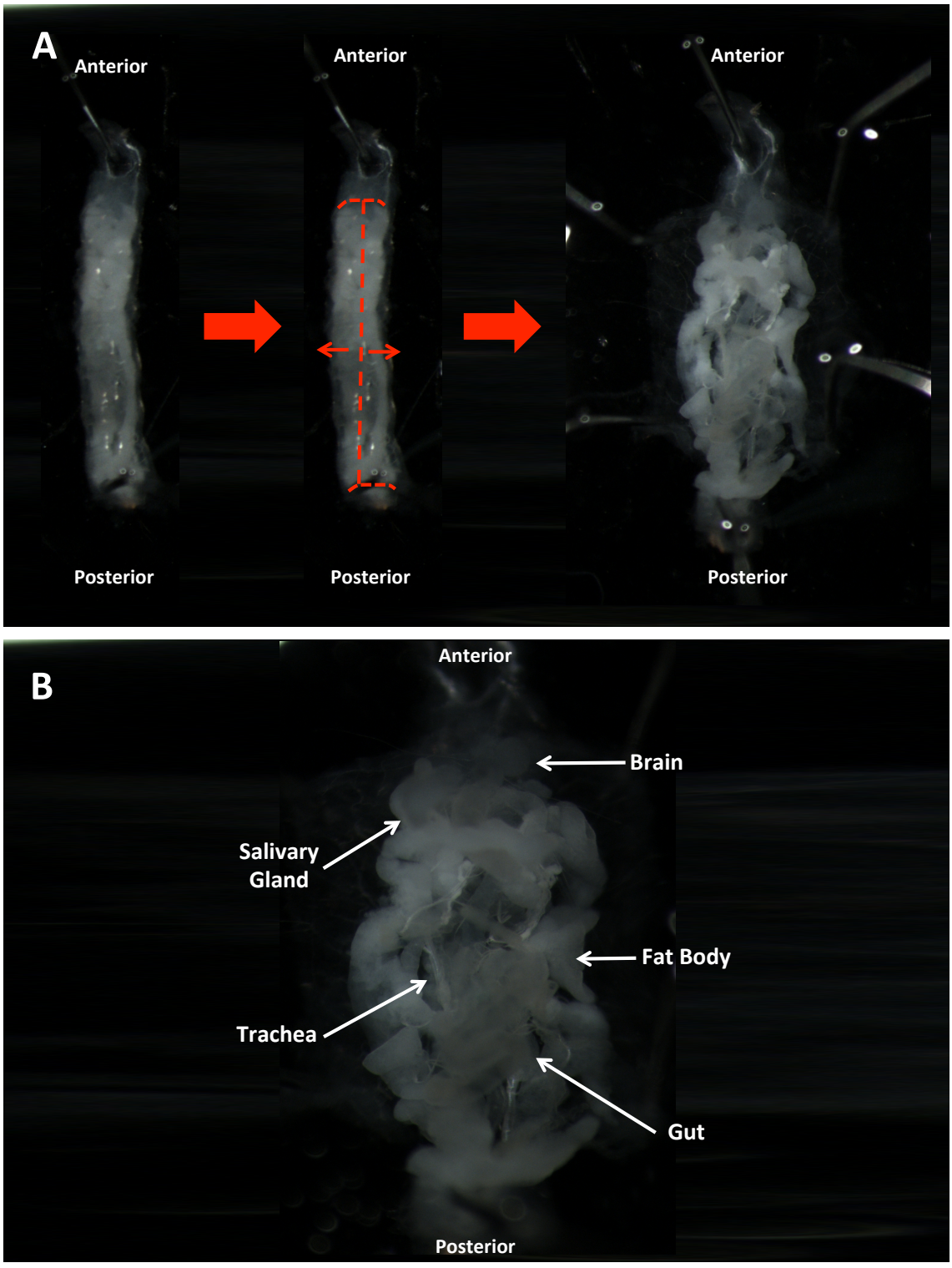


Figure 5-5: ***Dissection of 3rd instar larvae and anatomy of the internal organs.*** (A) Wandering 3rd instar larvae were picked and immobilised by pinning down at their anterior and posterior end. The larvae were opened in the vertical midline and fixed *in-situ* immediately.

(B) Schematic illustration of the anatomy of internal organs of a 3rd instar larva.

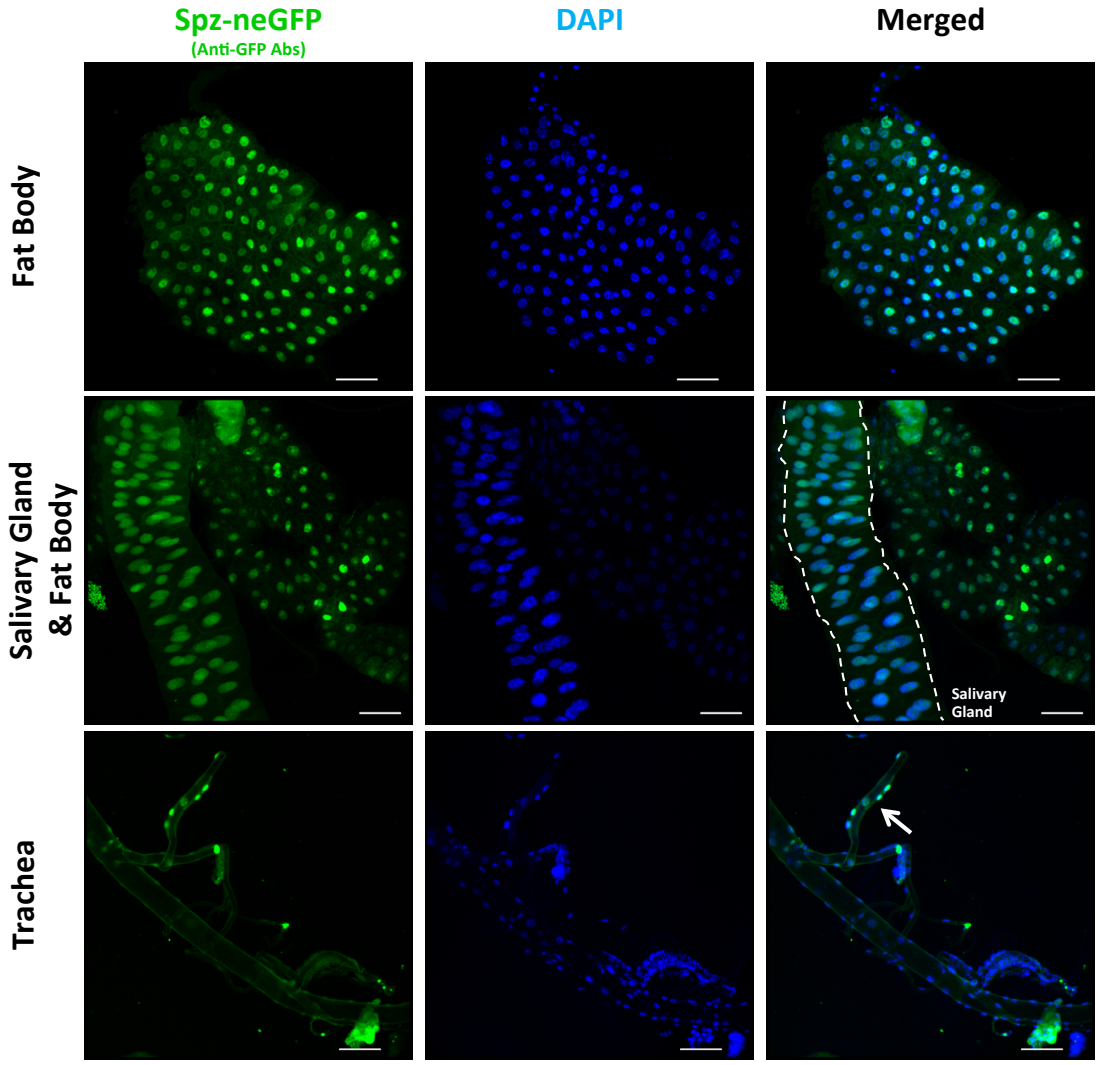


Figure 5-6: ***Immunostaining of dissected larval tissues revealed localised expression of the Spz-neGFP reporter.*** GFP expression was detected in the larval fat body tissues (top panel), the salivary glands (middle panel), and the bunching tracheas but not the main trachea (bottom panel) in 3rd instar larva (Scale bar: 100μm).

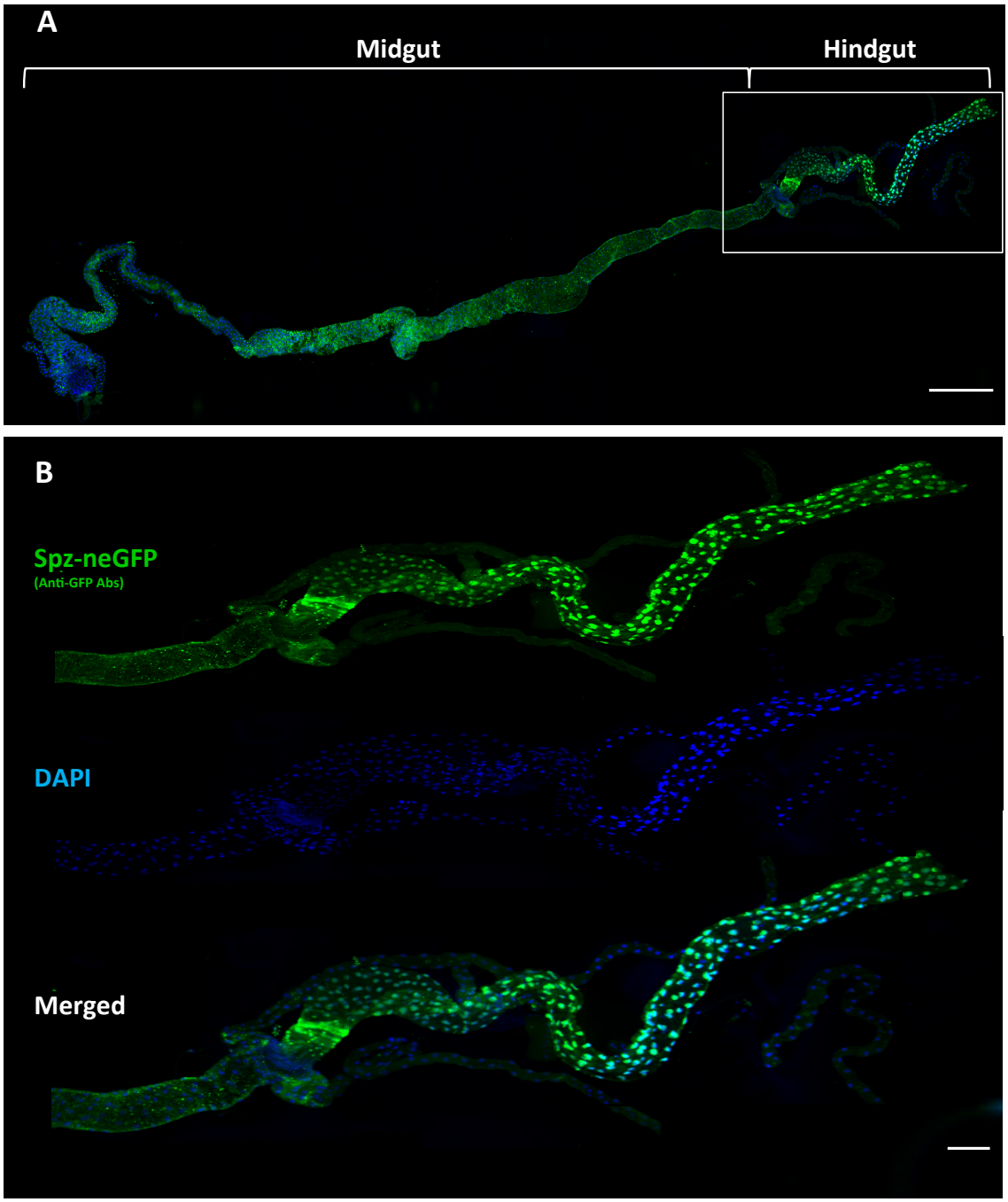


Figure 5-7: ***Immunostaining of whole 3rd instar larval digestive tract.***

(A) Dissected gut of a 3rd instar larva was stained with anti-GFP antibody and revealed expression of GFP in the hindgut, possibly by the intestinal epithelial cells (Scale bar: 500µm).

(B) Magnification of the hindgut and separation of the GFP and DAPI channel (200µm).

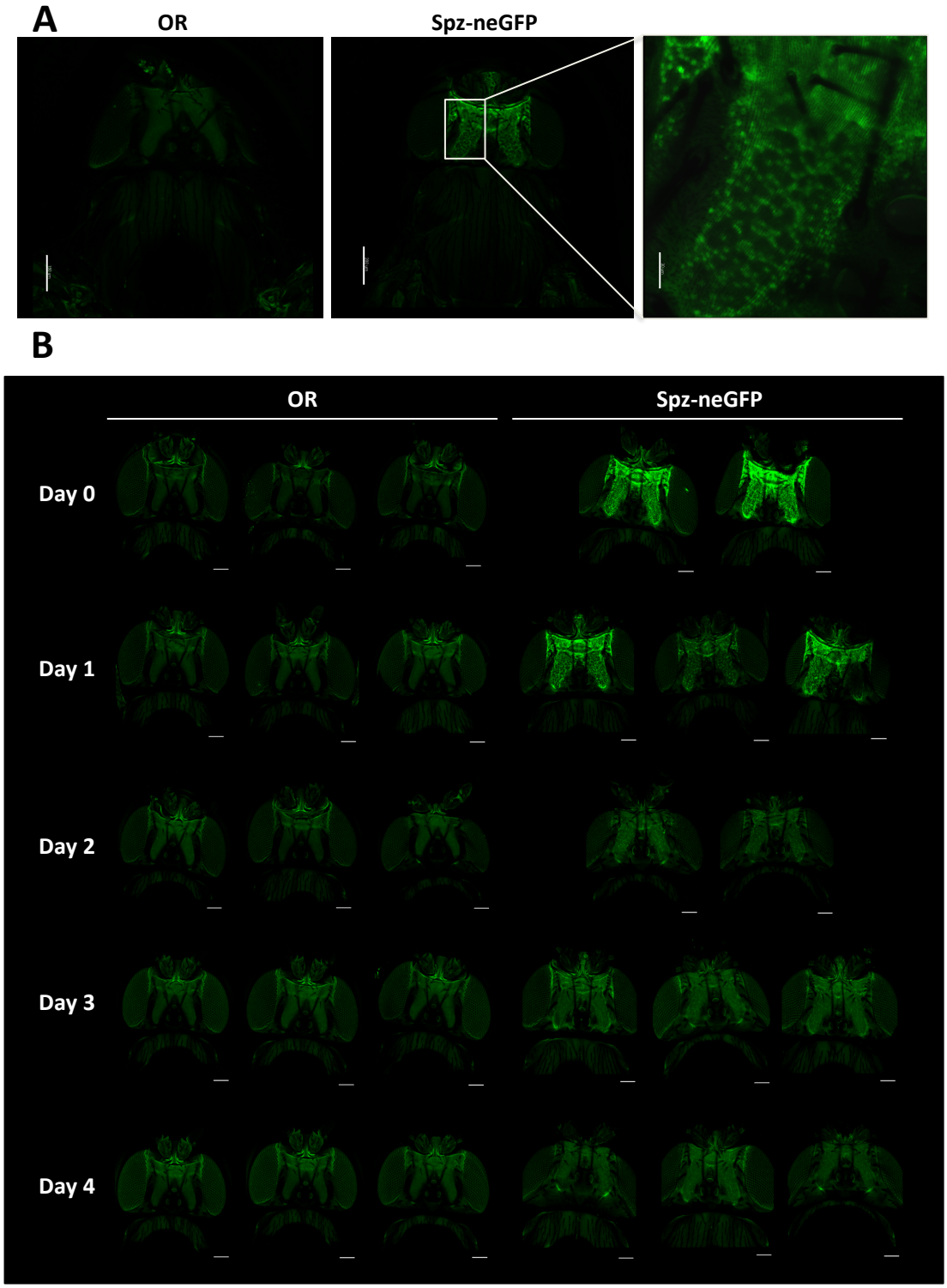


Figure 5-8: ***Localised expression of Spz-neGFP reporter gene at the head of young adult male flies.***

(A) GFP expression was detected in the head of a two days old adult male covering the dorsal side of the head except the ocellar triangle. A network pattern could be

observed in the area surrounding the ocellius (Scale bar – OR: 200μm; Spz-neGFP: 200μm; Spz-neGFP zoomed: 30μm).

(B) The fluorescence intensity was gradually declining in the head in the Spz-neGFP transgenic reporter flies. By four days after hatching, all GFP signal completely disappeared (Scale bar: 100μm).

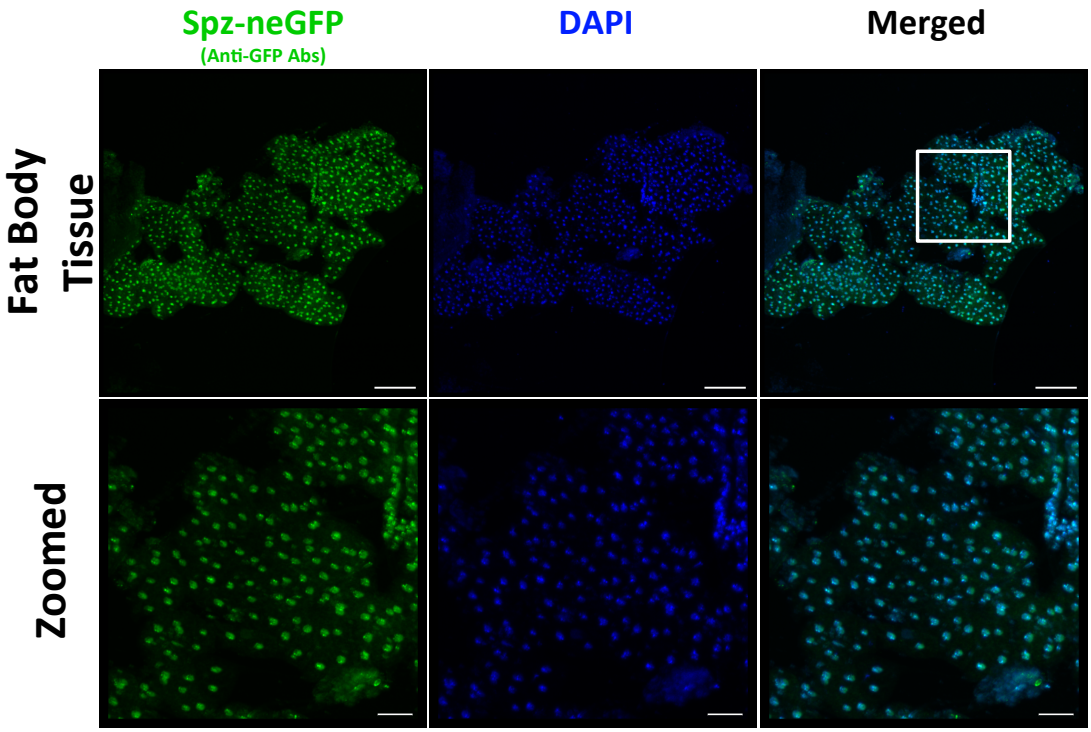


Figure 5-9: ***Immunostaining of dissected adult fat body tissues.*** GFP expression by adult fat body was revealed by immunostaining of the tissues with anti-GFP antibody (Scale bar – Top panel: 100µm; Bottom panel: 30µm).

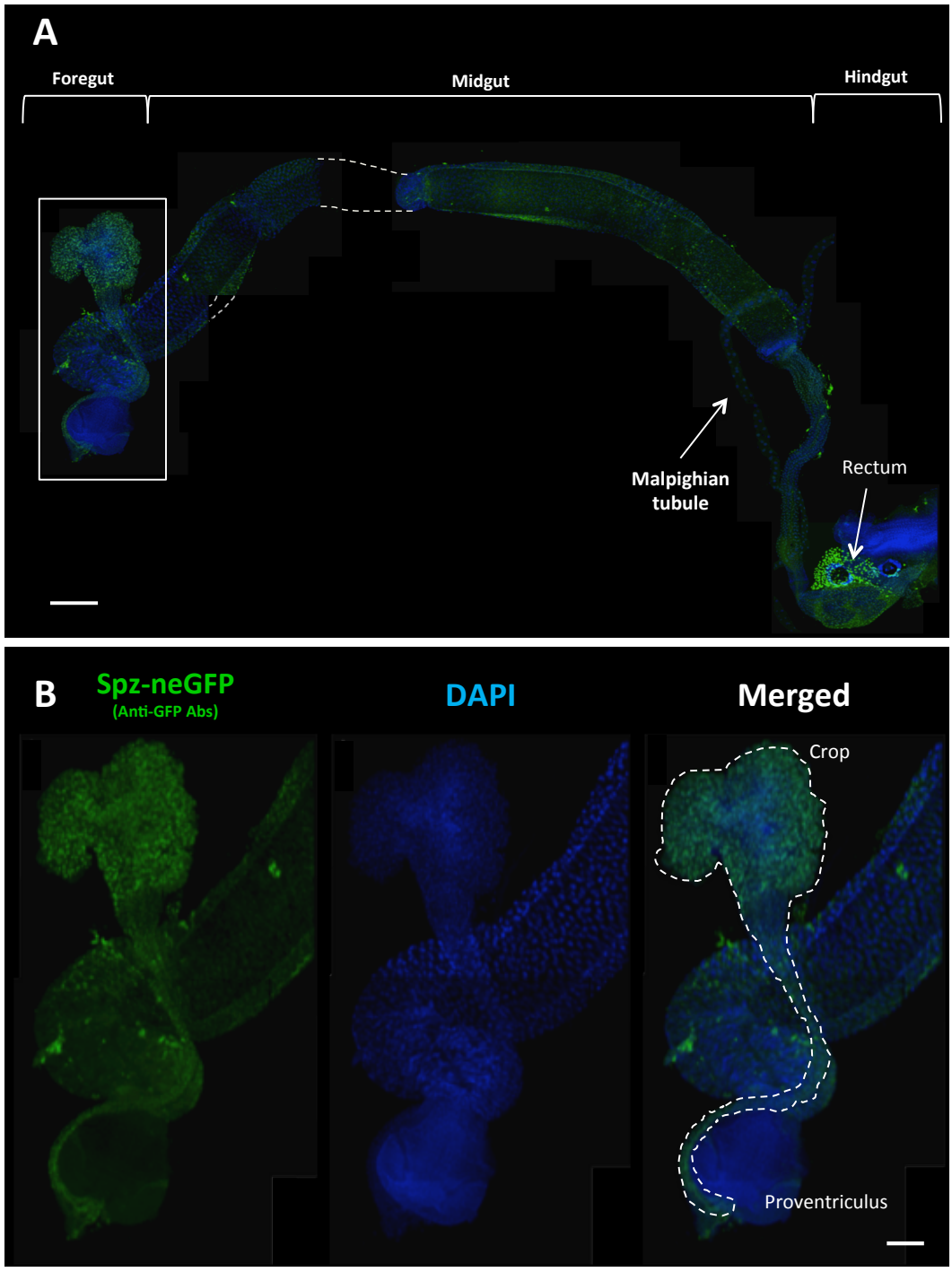
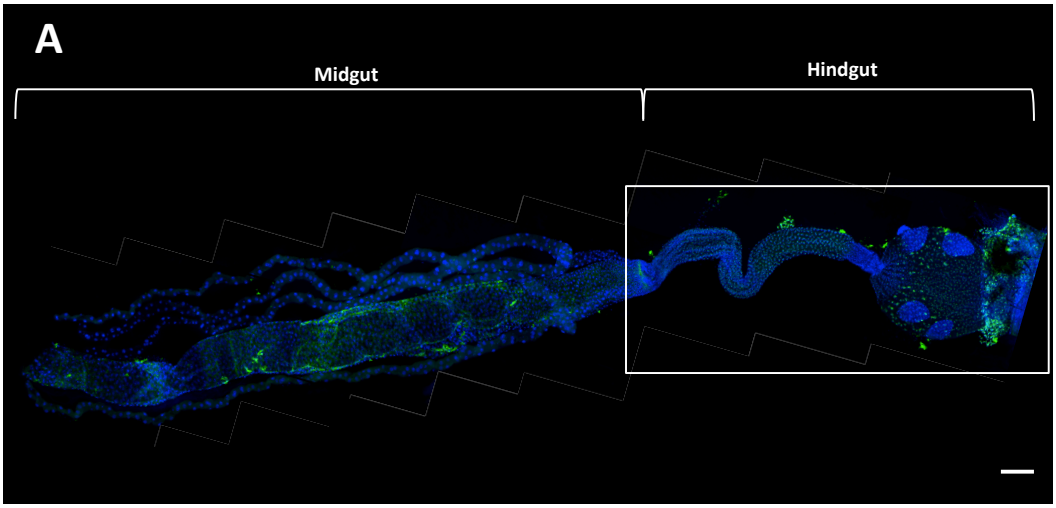
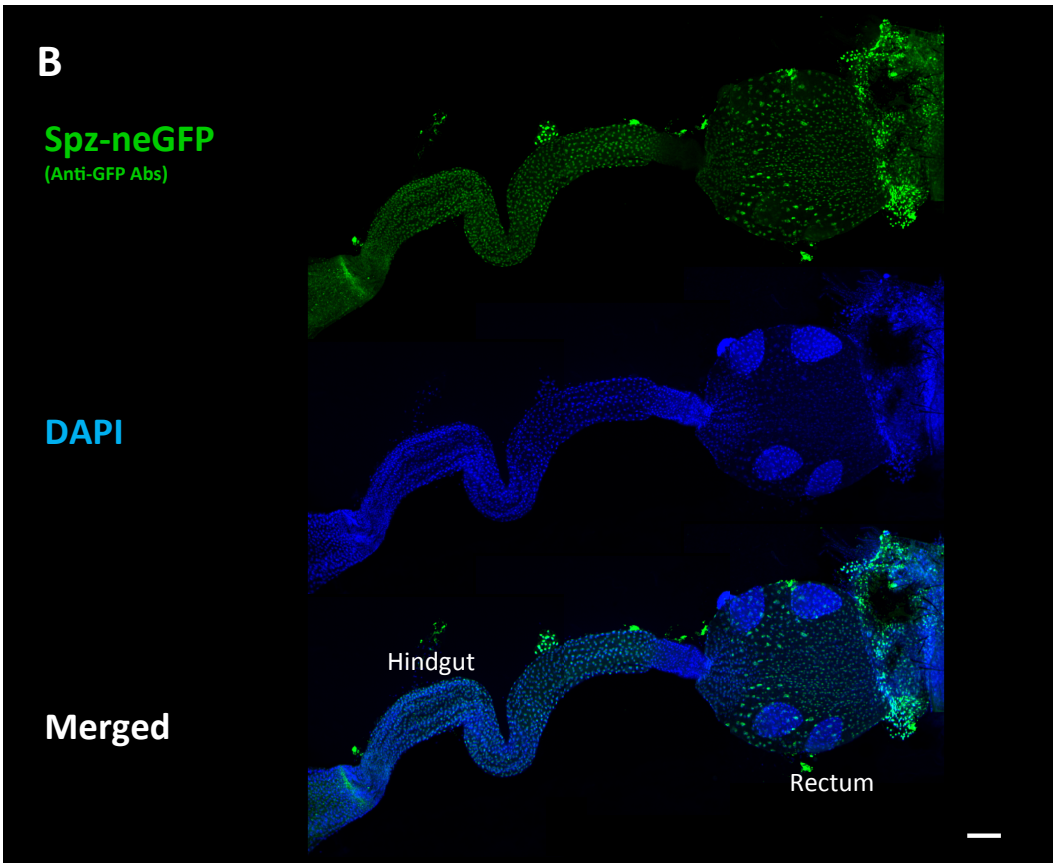


Figure 5-10: ***Immunostaining of whole adult digestive tract including the crop.*** (A) 10 days old male adult were picked and dissected to perform immunostaining. GFP expression in the crop and intestine was labelled by anti-GFP antibody. During the staining procedure, small part of the midgut epithelium was damaged and exposed the content. The damaged part is represented by white dash lines. GFP expression in the crop, hindgut and rectum was detected (Sacle bar: 200μm).

(B) Magnification of the foregut and separation of the GFP and DAPI channel. The shape and structure of the crop is shown by white dash lines (Scale bar: 100µm).


A
Midgut
Hindgut


B
Spz-neGFP
(Anti-GFP Abs)
DAPI
Merged
Hindgut
Rectum

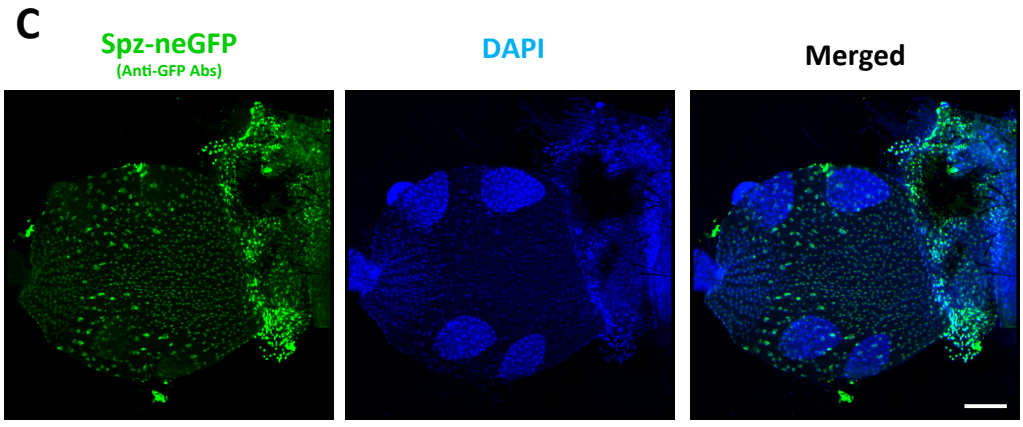

C
Spz-neGFP
(Anti-GFP Abs)
DAPI
Merged

Figure 5-11: ***Immunostaining of adult midgut and hindgut.***

(A) Immunostaining with anti-GFP antibody to reveal expression of green fluorescence in the hindgut and rectum (Scale bar: 500μm).

(B) Magnification of the hindgut and rectum and separation of the GFP and DAPI channel (Scale bar: 100μm).

(C) Magnification of the rectum and separation of the GFP and DAPI channel (Scale bar: 100μm).

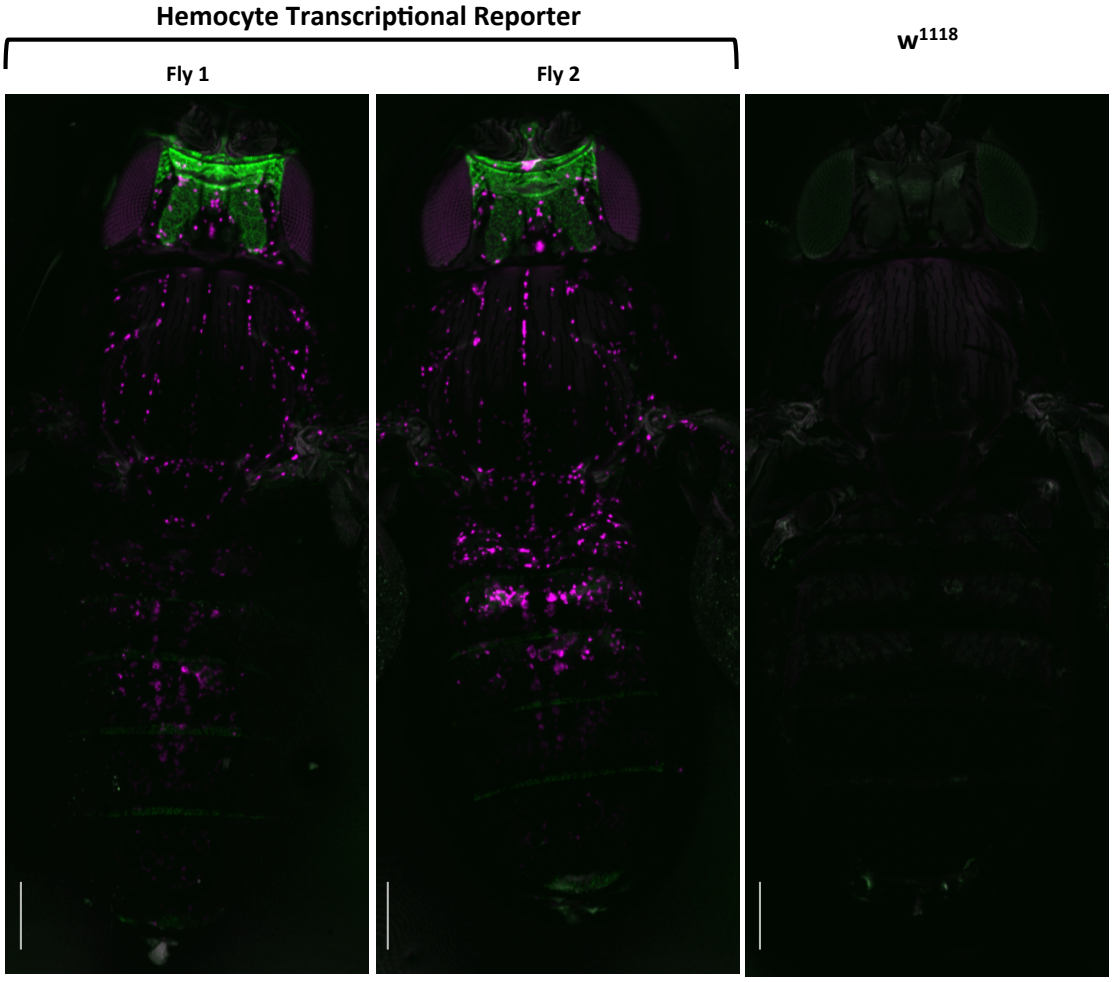


Figure 5-12: ***Confocal imaging of Hemocyte Transcriptional Reporter (w;Spz-neGFP;Crq-Gal4,UAS-CD8-Cherry/TM6c,Sb[1]).*** Young adult males (less than 5 days old) were picked and immobilised on its back by superglue during the imaging process. Green channel represents expression of the Spz-neGFP transgenic reporter, magenta represents adult hemocytes while age matched w^{1118} male fly was used as the wild type control (Scale bar: 200μm).

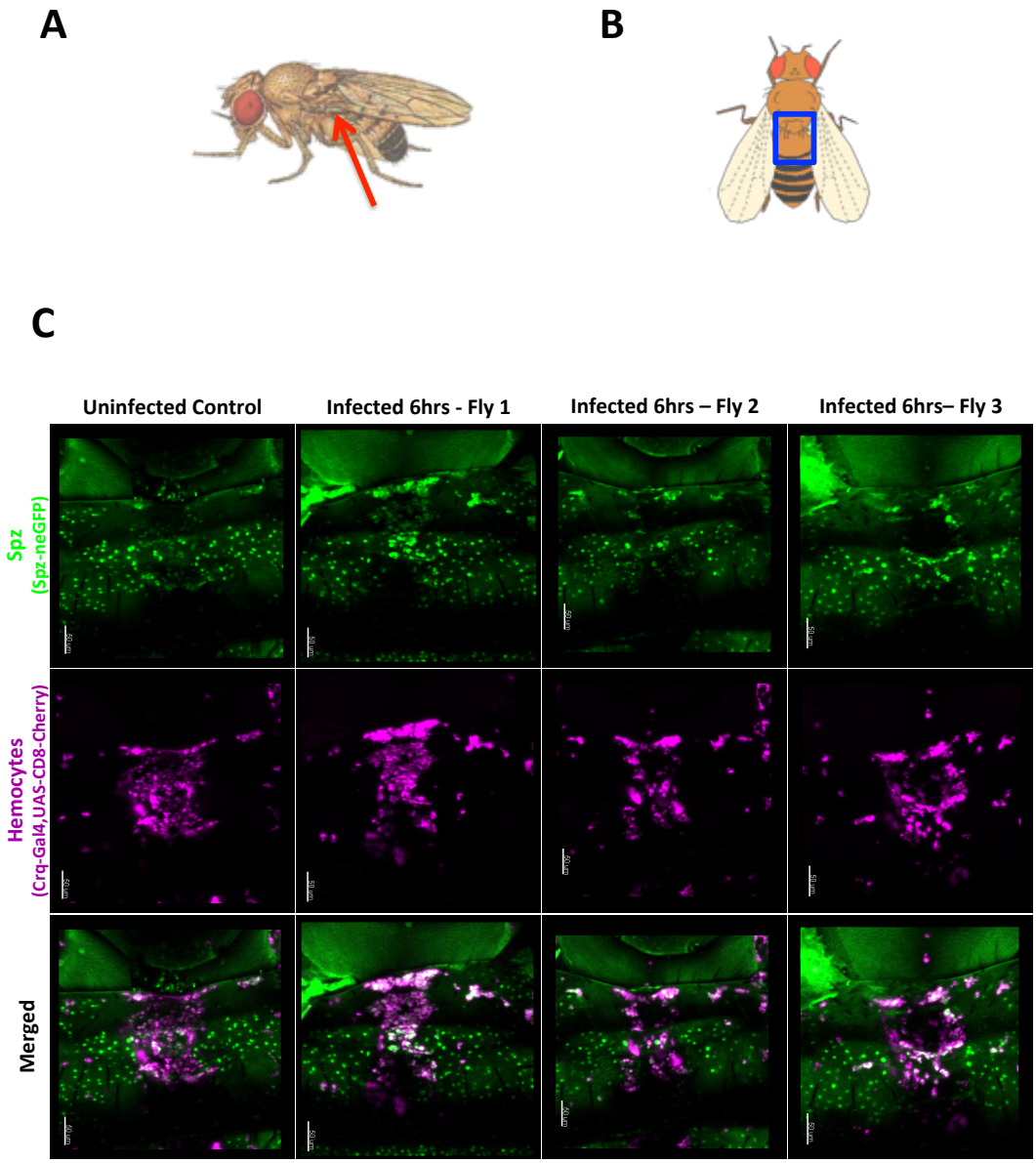


Figure 5-13: ***Induction of the Spz-neGFP transgenic reporter in hemocytes following infection with bacterial mixture.***

(A) To immunize, adult male *Drosophila* of 10 days old were collected for bacterial injection. During injection, flies were anesthetised with CO_2 and the needle pre-filled with bacteria would penetrate the fly surface at the side of upper abdomen between the connection of the dorsal and ventral cuticle (pointed by red arrow).

(B) After injection, flies were allowed to recovered at 25°C for 6 hours before *in-vivo* imaging with confocal microscopy. For imaging, individual fly was immobilised on its back on a cover slip by superglue as the diagram shown. The

blue square indicates the area around the first heart chamber where the imaging was taken place.

(C) GFP induction in adult hemocytes located in the heart was observed following bacterial immunization. GFP expression in non-hemocytic cells and in small numbers of hemocytes was detected in fly without infection. Upregulation of green fluorescent in adult hemocytes was recorded 6 hours post injection with the bacteria mixture containing *E. coli* and *M. luteus* (Scale bar: 50μm).

- Chapter 6 -
Discussion and Future Perspective

6.1 The Spz Expression Profile

6.1.1 Expression of the Spz-neGFP transgene in embryonic hemocytes

6.1.1.1 Expression of Spz in embryonic plasmatocytes could be for immunological purpose

In embryos, expression of the Spz-neGFP transgene was observed in hemocytes at embryonic stage 14 when plasmatocytes begin to migrate out of the head region to populate the entire embryo. The function of Spz expressed in hemocytes at this stage is not yet clear. Possibly, it could be providing important assistance to the embryonic hematopoietic process including hemocyte development, survival, and migration, as well as providing protection against infection.

Since the fat body are not mature until embryonic stage 16 to 17, we therefore believe that hemocytes are the major source of immune responses before the fat body is fully developed. Immune challenge with Gram-positive bacteria or fungus in *Drosophila* leads to activation of the Toll pathway. Spz is the known ligand for Toll for both development and immune responses. Following detection of bacterial or fungal components, pro-Spz is activated through enzymatic cleavage mediated by the serine protease Spz-processing enzyme (SPE). The protease SPE is expressed in the developing fat body and lymph glands from as early as the embryonic stage 11 (Mulinari et al., 2006). In the transgenic Spz-neGFP reporter flies, GFP signal was detected in the putative epidermal cells from the embryonic stage 11 onwards, which appears to correspond with the expression timeline of SPE. Since the fat body tissues are not fully functional until the later stages of

embryogenesis, it is possible that mature embryonic hemocytes (both plasmatocytes and crystal cells) are the main, and possibly the only, immunological organ to protect the embryo from pathogens and any immune challenge. Since Spz is being constitutively expressed by the fat body during larval stages (Irving et al., 2005), it is possible that Spz is also constitutively expressed in embryonic hemocytes as soon as the hemocytes become mature and lasts until the fat body tissues develop and take over.

6.1.2 A new reservoir for Spz production – the epithelial cells and the hindgut

In addition to embryonic hemocytes, expression of the Spz-neGFP transgene was observed in the putative embryonic epithelial cells from stage 11, and its expression persisted into the pupal stage. The local immune response in the epidermal cells was reported to be Spz/Toll independent (Ferrandon et al., 1998). Using a Drosomycin-GFP reporter, it has been shown that expression of the antimicrobial peptide Drosomycin by the epithelial cells was not affected in a Toll or Spz loss-of-function mutant background. This indicates that production of Drosomycin in response to immune challenge was not due to systemic activation of the Toll signalling cascade but to a localised event. Using the transgenic Spz-neGFP reporter, Spz was constitutively expressed in the epithelial cells without the presence of an immune stimulus. This finding suggests that, together with the fat body, the epithelial cells could be an additional source of Spz synthesis. Together with previous studies where Drosomycin production by the epithelial cells had been shown to be Spz/Toll independent (Ferrandon et al., 1998), our Spz

expression data suggests that epithelial cells may synthesise and secrete Spz as part of the systemic immune response. It could be involved in systemic reaction through communication with other immunological organs such as hemocytes and the fat body.

The hindgut is a novel organ that has been identified to be expressing Spz in both 3rd instar larvae and in adult in the absence of infection. Oral infection with an isolate of *Erwinia carotovora carotovora* (Ecc15), non-lethal Gram-negative bacteria, is able to trigger systemic immune responses including the Toll pathway resulting in production of antimicrobial peptide Drosomycin (Basset et al., 2000). However, no Toll activity has been detected in the gut upon oral infection with Ecc15 (Buchon et al., 2009). This is an interesting observation as the transgenic Spz-neGFP reporter has shown constitutive expression of green fluorescent in the hindgut where Toll activity has shown to be absent (Buchon et al., 2009).

Both epithelial cells and hindgut are organs that have contact with the surrounding environment – outside environment by epithelial cells and food ingested by the hindgut – and protect the flies against potential infectious agents and damage, such as wounding. It is thus reasonable to speculate that these organs are capable of regulating not only the local immune response, but also the systemic reaction against immune challenge. However, the mechanism employed by epithelial cells and the hindgut to modulate systemic immune response is poorly understood. As one of the most essential signalling pathway in *Drosophila*, the Toll pathway protects the fly from fungal and gram-positive bacteria infection. Observation of Spz constitutive expression in these organs might provide us further insight into

regulation of systemic immunity by the Toll signalling cascade. It has been previously reported that Spz mRNA is expressed by larval fat body constitutively and circulating in the hemolymph as an inactivated form (Irving et al., 2005). These mRNA are serving as a reservoir for Spz and prepare the larvae for potential infectious or inflammatory event. Upon recognition of fungal or bacterial components, the inactivated Spz in the circulation can rapidly activated through a series of enzymatic conversion and signal to hemocytes for further production of Spz that subsequently trigger the Toll pathway in fat body resulting in antimicrobial peptides synthesis. Activation of the Toll pathway in the fat body by Spz secreted by hemocytes can also upregulate Spz expression to amplify the signal and hence forming a positive feedback loop. Yet, it is not clear what triggers this constitutive expression of Spz mRNA in fat body and whether it is linked with environment sensing.

Therefore, we hypothesise that both epithelial cells and the hindgut might feasibly function as a reservoir of Spz in the absence of immune challenge. Expression of Spz in these organs might be related to the surrounding environment and food sources. Besides, Spz expressed by epithelial cells and the hindgut might hold a possible role in communicating with the fat body to support the constitutive expression of Spz mRNA in the absence of infection or inflammation (Fig. 6-1). When experiencing an immune challenge in the epithelia layer such as wounding, or an infection in the gut, epithelia cells and the hindgut might trigger extra transcription of Spz and secrete the cytokine to activate the systemic immune response through signalling to the hemocytes and fat body. To confirm our

hypothesis, further studies are required and detail plan to accomplish this aim will be discussed in Section 6.3.1.

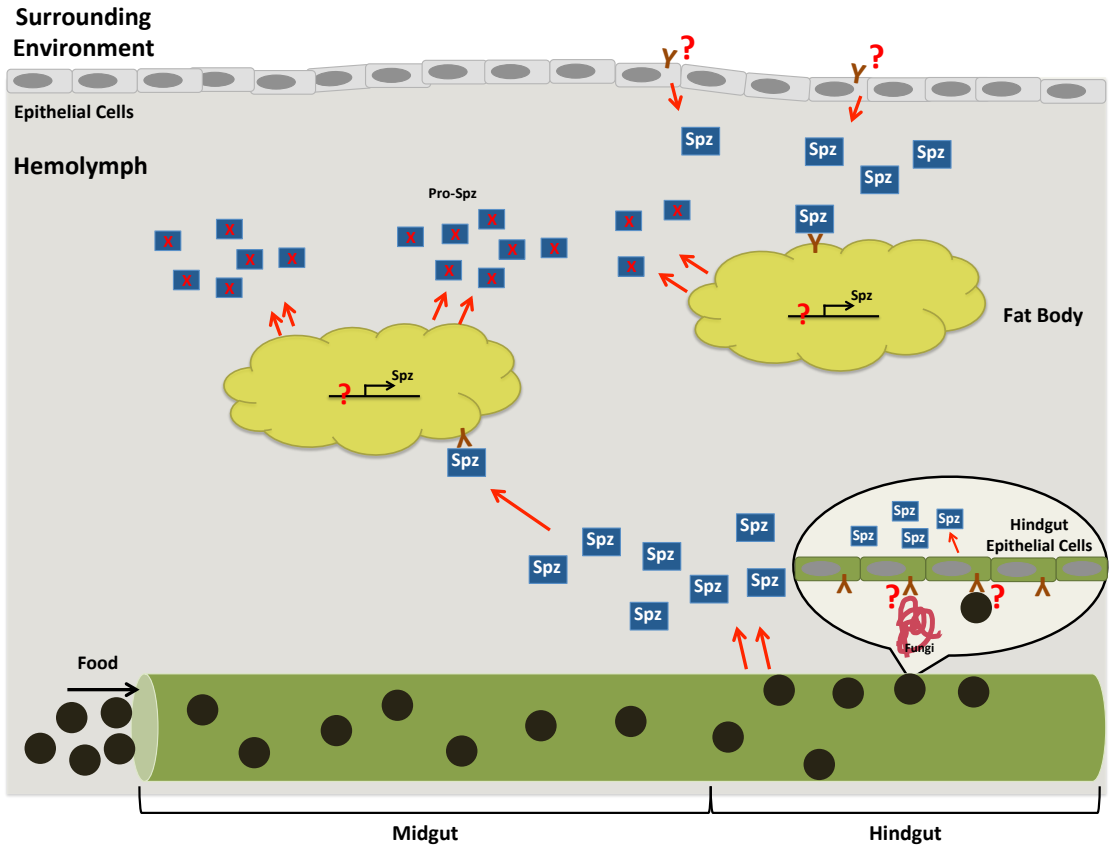


Figure 6-1: ***Two new reservoirs for Spz production and a proposed mechanism in cross communication between these reservoirs and the fat body.*** Using the novel Spz-neGFP transgenic reporter, we have identified two new tissues – the epithelial cells and the hindgut – that express Spz constitutively without the presence of immune stimuli. These two tissues could be feasibly acting as a reservoir for Spz production and trigger systemic Toll responses through communicating with the fat body and hemocytes upon detection of pathogens.

6.2 Summary

In the past two decades, *Drosophila* has been used to study innate immune response and has lead to many major breakthroughs including discovery of the antifungal role of the Toll pathway. However, there are still many aspects that require further characterisation and understanding. For example, *Drosophila* hemocytes are known to possess an important role in phagocytosis following detection of pathogenic agents in the fly body. Recent findings also suggested that *Drosophila* hemocytes are capable of secreting cytokines – in a manner that highly resembles the functions of mammalian monocytes and macrophages – to regulate systemic immune response as a result of immune challenges, such as bacterial or fungal infection and wounding. Activation and regulation of cytokines transcription upon different types of immune challenges in hemocytes is not yet thoroughly characterised. Hence, the aim of this study is to obtain further insight and better understanding on the transcriptional control of different *Drosophila* cytokines, including Spätzle (Spz), Unpaired3 (Upd3) and Eiger (Egr), upon infection and inflammation. To achieve this aim, a genome wide *in vivo* screen that employs both fluorescent hemocytes and cytokines reporter and RNAi lines will be developed. In addition, this screening system will also allow us to identify potential upstream transcriptional regulators for these cytokines and their downstream signalling pathway. During the screen, *Drosophila* hemocytes are labelled by red fluorescent protein (RFP) using the Gal4-UAS expression system whereas endogenous cytokine expression is reported by green fluorescent protein (GFP) knocked in to the locus of individual cytokine. The Gal4 transcription factor linked

with hemocyte reporter will also be used to drive expression of the RNAi lines to ensure tissue specific knock down effect.

We attempted to generate GFP knock in reporter for Spz, Upd3 and Egr using the ends out replacement techniques documented in previous publication (Huang et al., 2008). The knock in process can be separated into three different stages - 1) generation of cytokine knock in plasmid; 2) transgenic stage with the knock in plasmid randomly inserted into the fly genome; and 3) the knock in stage which targeted cytokine locus is replaced by GFP through homologous recombination. Out of the three cytokines, we have successfully generated a Spz-GFP reporter flies at both transgenic and knock in stages. Due to the complex cloning process for generation of cytokine knock in plasmid and the intensive time and labour required for the knock in process, we have decided to focus solely in setting up the *in vivo* genome wide screen with the available Spz-GFP transgenic and knock in reporter. Three forms of eGFP, eGFP, 2xeGFP (eGFP-IRES-eGFP) and neGFP (nuclear eGFP), were used to generate the Spz-GFP knock in plasmid and all three plasmids were inject into the fly genome to generate the Spz-GFP transgenic reporter. GFP expression in these reporter flies at the transgenic stage was examined and only Spz-neGFP transgenic reporter display GFP signal.

Out of the three Spz-GFP transgenic reporter flies, we have successfully generated a novel Spz knock in reporter from Spz-eGFP transgenic flies. However, no GFP signal was detected in both heterozygous and homozygous knock in reporter and thus making the knock in reporter an insufficient tool for the screen. Phenotypic characterisation of the homozygous Spz-eGFP knock in reporter, also a null mutant

of Spz, was conducted. Unlike other Spz mutants, no developmental defect was detected in this Spz null mutant as sufficient Spz mRNA remained to be placed in the embryo of the null mutant by its heterozygous mother. Upon infection with fungus *C. albicans* and bacteria *M. luteus* and *E. coli*, the Spz null mutant displayed a trend of significant life span reduction.

Since the Spz-eGFP knock in reporter is an insufficient imaging tool, we have explored the possibility of using Spz-neGFP transgenic reporter as a faithful replacement for the *in vivo* genome wide screen. Expression of the Spz-neGFP reporter gene was controlled by the Spz 5' and 3' homology regions directly cloned from whole fly genomic DNA. Through characterisation of the transgenic Spz-neGFP reporter flies, the expression pattern of Spz during different developmental stages was revealed and appeared to be matching with previous documentation of Spz expression. Additionally, we have observed expression of Spz by the larval epithelial cells in and the hindgut in both larvae and adult. These locations might be serving as a reservoir for Spz production and might have a possible role in regulating the systemic immune responses through communication with other tissues such as hemocytes and fat body by secreting Spz. Hence, the transgenic Spz-neGFP reporter can be a faithful reporter for the *in vivo* screening system and will potentially be a useful reporter to provide more insight into Spz biology including when and where the *Spz* gene is being transcribed during development as while as undergoing an immune challenge.

In conclusion, we have successfully generated hemocyte reporters, Spz transgenic reporter and Spz knock in reporter for the genome wide *in vivo* screening that will

be used to decipher the transcriptional regulation of Spz in hemocytes during infection and inflammation. We have also characterised the Spz-neGFP transgenic reporter and established a Spz expression profile from embryonic stage to adult. By challenging this transgenic reporter with a bacteria mixture composed of *M. luteus* and *E. coli*, we obtained the preliminary view of Spz expression upregulated in hemocytes as a consequence of bacterial infection. Last but not least, we have conducted preliminary characterisation on the immune response of Spz-eGFP null mutant and observed a similar phenotype as reported previously (Lemaitre et al., 1996) following bacterial and fungal infection. With these reporters, we are now ready to develop the screen and the set up will be further discussed in the next section.

6.3 Future Perspective

6.3.1 Further characterisation of Spz and its role in local and systemic immunity

To confirm our hypothesis that Spz secreted by epidermal and hindgut might be acting at a systemic level, via signalling to the fat body and hemocytes, different experimental approaches will be employed and tested.

Currently, the stimuli that trigger the constitutive expression of the Spz-neGFP transgene in the epidermal cells and hindgut in the transgenic reporter flies have not yet been identified. Thus, it is important to isolate the candidate stimulus that elicits the continuous expression of the transgene in these cells. Flies raised in a laboratory are kept on food that is supplemented with yeast, which is known to activate the Toll signalling pathway. Therefore, it is necessary to assess potential changes in GFP expression in Spz-neGFP transgenic reporter flies that are raised on a yeast-free food. Comparing the GFP intensity between transgenic reporter flies grown on a yeast-containing or yeast-free diet might allow us the first glimpse of if and how yeast modulates expression of the Spz-neGFP transgene in specific populations of cells.

Between the two distinct tissues – the epidermal cells and the hindgut – that are shown to be expressing the Spz-neGFP transgene, we are particularly interested in studying Spz expression in the hindgut. As Toll activity is not required for immune response within the fly gut (Buchon et al., 2009), we propose that infection within the digestive tract, especially in the hindgut, might lead to activation of the

systemic immune responses through Spz synthesis in the hindgut. In a recent paper, it has reported that both Toll pathway and hemocytes play key roles in the activation of systemic immune responses during gastrointestinal infection with clinical isolates of *C. albicans* in *Drosophila* larvae (Glittenberg et al., 2011). Interestingly, activation of the Toll cascade in the fat body upon gut infection is suggested to be dependent on the serine protease Persphone (Psh), an alternative route than GNBP3-ModSP, to trigger Spz cleavage by SPE. In addition to the Toll pathway, hemocytes have also been shown to be essential to establish the systemic reaction post gastrointestinal infection, as providing the 'self-signal' to the fat body via a yet unknown mediator (Glittenberg et al., 2011).

The potential role of hindgut-specific Spz in systemic immune responses will be examined using fungus *C. albicans*. Transgenic Spz-neGFP reporter flies and controls will be infected with the fungus orally. The GFP signal and its intensity will be examined by confocal microscopy and quantitative PCR. Besides, the immune responses in the Spz^{eGFP} null mutant will also be extensively examined following gastrointestinal infection with *C. albicans*. In parallel, Spz expression in the digestive tract will be conditionally knocked down using the gut-specific Gal4 driver in a wild type background. By monitoring the Spz level in the hindgut and fat body tissues during steady and inflammatory condition using quantitative PCR, it will allow us to achieve preliminary vision into the complex regulations between the gut and systemic immunity.

6.3.2 Application of the transgenic Spz-neGFP reporter – a Genome Wide Screen

Following further analysis of the novel Spz-neGFP transgenic reporter flies, the goal is to develop a genome wide screen by employing the exhaustive UAS-RNAi library available. The aim of the screen will be to identify new candidate gene(s) and/or pathway(s) that regulate the transcription of *Spz*, with specific interest in hemocytes, in both positive and negative manner upon immune challenge in adult *Drosophila*.

The UAS-RNAi flies will be obtained from the two existing RNAi libraries located in Japan and Austria; the combined libraries cover 92% of the fly genome. The RNAi flies will be crossed to appropriate Hemocyte-Gal4 drivers to generate the hemocyte transcriptional reporter fly – flies that are deficient in a given gene, as well as carrying *Spz* labelled with GFP, and hemocytes with red fluorescent protein. The knock down effect of the RNAi flies will be restricted in hemocytes in the F1 progenies as the UAS-RNAi lines will only be driven by Hemocyte-Gal4 ensuring tissue specific gene silencing. Using both confocal microscopy and quantitative PCR, the transcription level of *Spz* can be assessed after immunising with appropriate pathogens.

The screen will be conducted via two approaches – candidate gene approach followed by an open approach. A small-scale candidate gene based screen will first be developed to optimize and validate the screening system. As previously suggested, constitutive expression of Spz mRNA by the fat body might be the

consequence of Toll activation, and hence leading to the formation of a positive feedback loop of *Spz* (Irving et al. 2005). To examine this hypothesis, genes that are involved in the Toll cascade will be selected for the initial candidate gene screening.

In the open approach, we are aiming to screen all available UAS-RNAi lines in order to identify potential new regulatory candidate(s) for the Toll signalling pathway. Due to the large amount of genes that will be covered at this stage, the genome wide screen will be conducted in a high throughput setup using confocal microscopy (Fig. 6.2). After challenge with appropriate pathogens, the transcription level of *Spz* – represented by GFP intensity – with individual candidate gene(s) being silenced through hemocyte specific knock down – will be monitored and quantified by confocal microscopy (Fig. 6.2). Candidate gene(s) that are able to modify Spz transcription post-infection will be selected and confirmed using quantitative PCR.

Since the *Drosophila* Toll and mammalian Toll-like receptor signalling pathways are highly conserved, we are hoping to be able to translate any novel candidate(s) identified through this genome wide screen to the mammalian immunity, and eventually help define new therapeutic directions and strategies for inflammatory diseases.

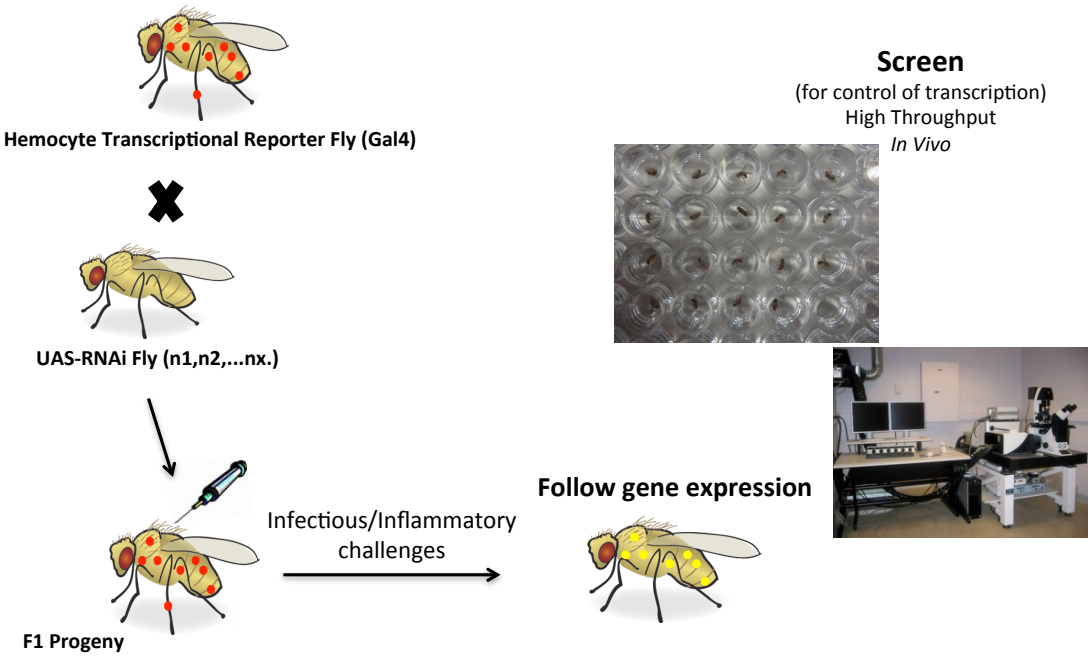


Figure 6-2: ***Set up of the genome wide screen to identify potential candidates that are involved in the regulation of Spz transcription in hemocytes.*** By crossing the hemocyte transcriptional reporter fly with the USA-RNAi fly, the knock down effect of the RNAi line will be specific to hemocytes. Gene expression will be followed in the F1 progeny following immune challenge with appropriate pathogens. To run the screen in a high throughput manner, multiple flies will be screened, initially on the changes on the GFP level, by confocal microscopy.

- Chapter 7 -
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Supplementary Figures

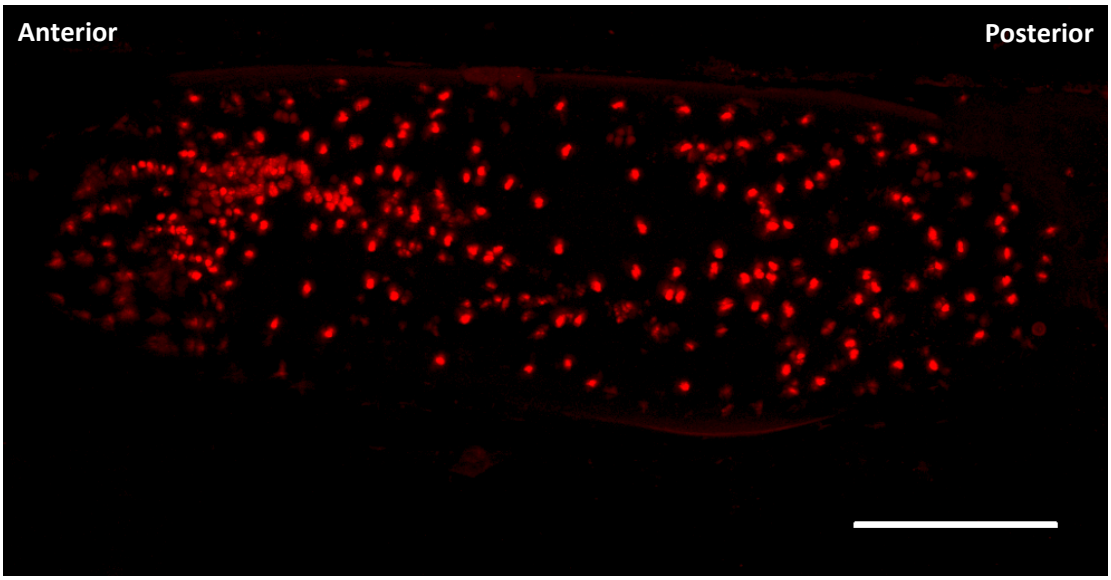


Figure 1: ***Visualisation of embryonic hemocytes.*** A stage 15 embryo of w;Sn-Gal4,UAS-RedStinger/Cyo was prepared and mounted in low-melt agarose during in-vivo imaging with confocal microscopy (Scale bar: 100µm).

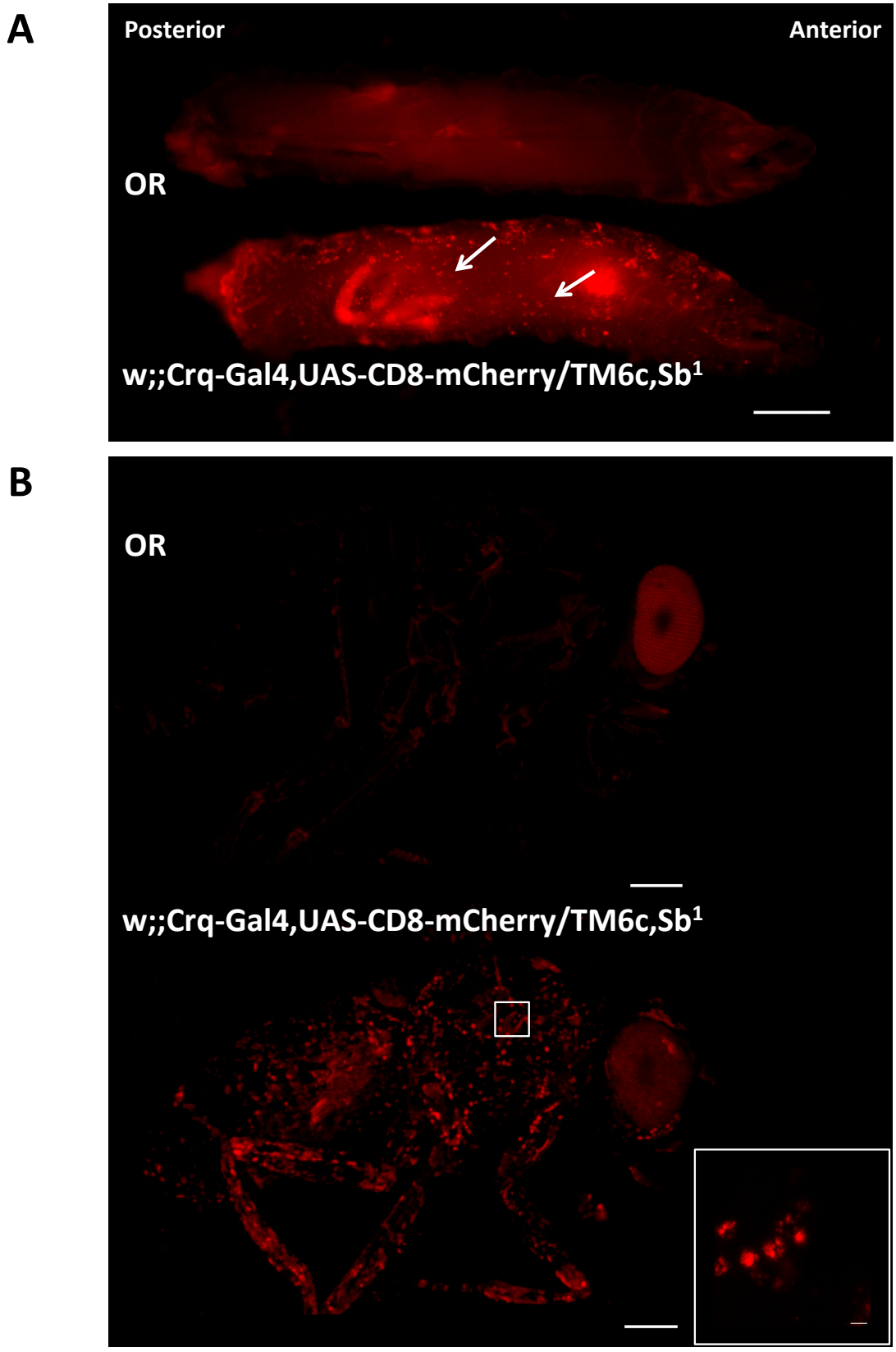
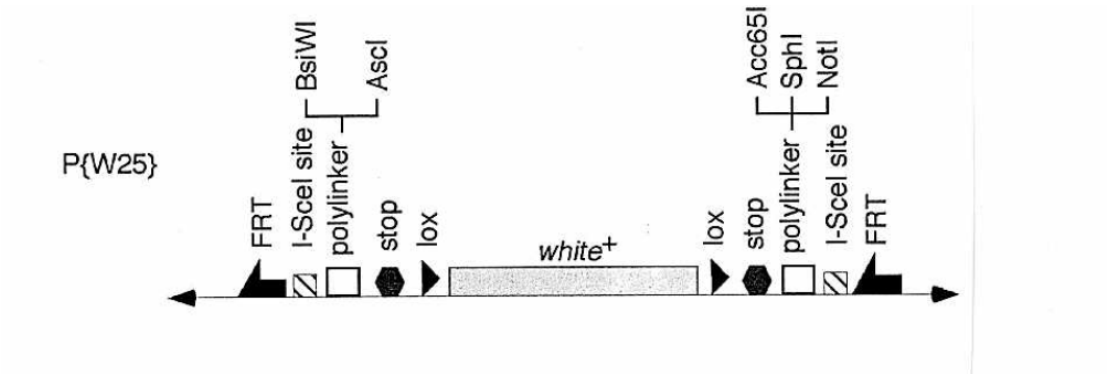


Figure 2: ***Visualisation of larval and adult hemocytes using a membrane-bound-RFP hemocyte reporter.***

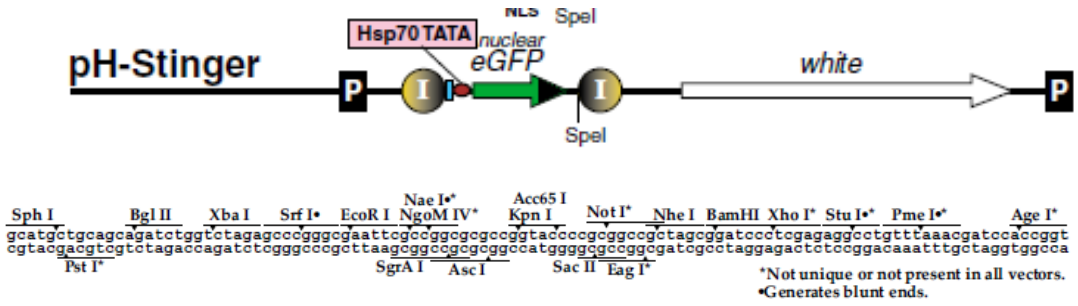
(A) Wandering 3rd instar larvae of OR and w;;Crq-Gal4,UAS-CD8-Cherry/TM6c,Sb^1 were picked for intravital imaging with fluorescent microscope. The larvae were first washed with sterile PBS and were immobilised using ice-cold PBS during imaging (Scale bar: 500μm).

(B) 7 days old male adult of OR and w;;Crq-Gal4,UAS-CD8-Cherry/TM6c,Sb^1 were picked and imaged with confocal microscopy. During the imaging process, the flies were anesthetized with CO_2 (Scale bar: 200μm; Insertion: 30μm).

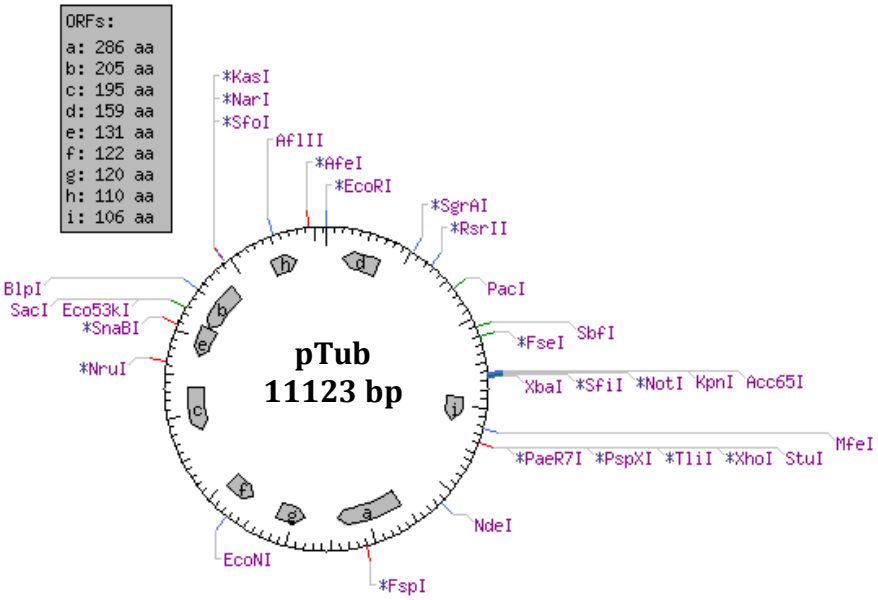
Appendix



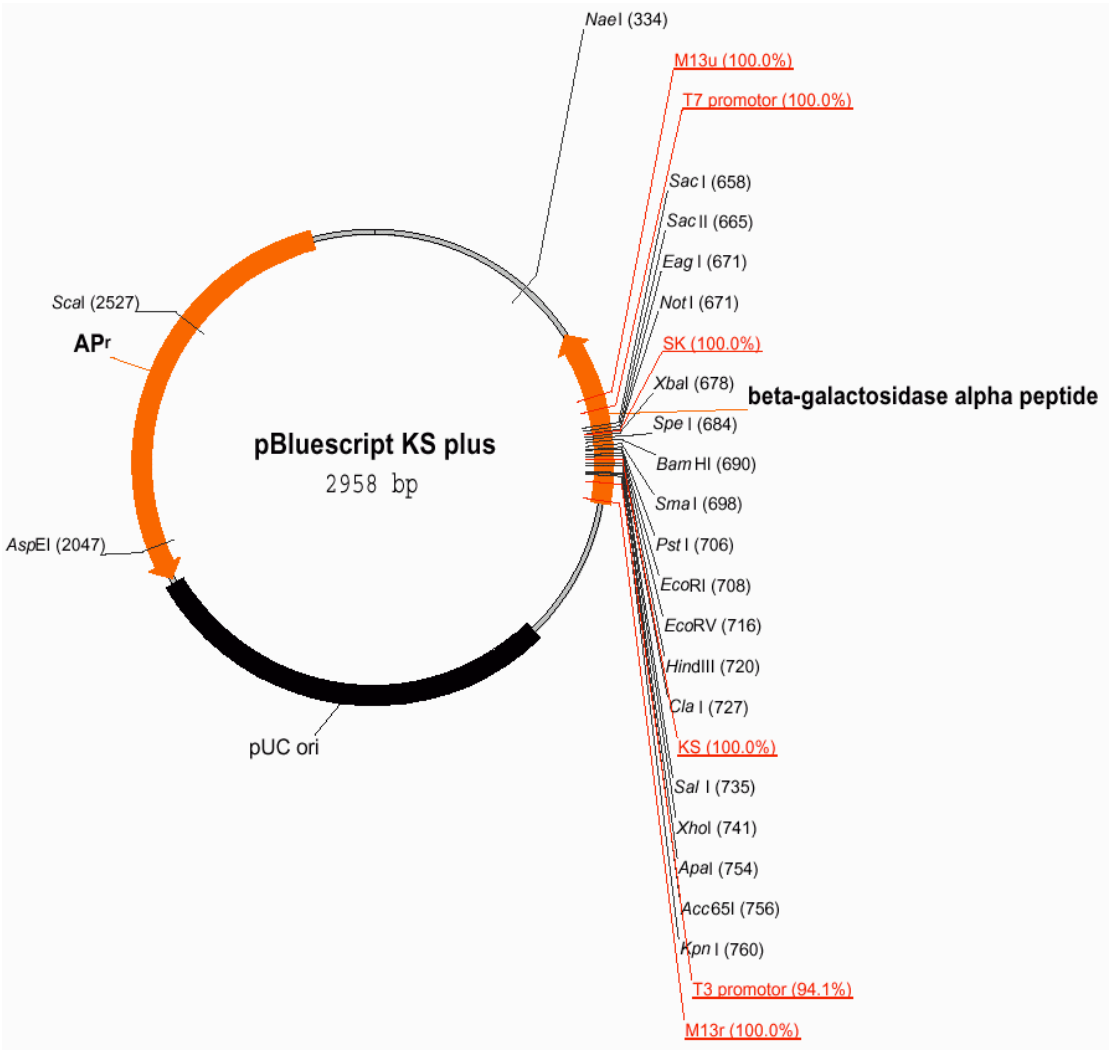
Appendix 1: ***The map of the pW25 plasmid showing the multiple cloning sites and the FRT I-SecI sites decided for homologous recombination.***



Appendix 2: ***The map of the pH-Stinger plasmid.*** The nuclear eGFP (neGFP) fragment was obtained from the pH-Stinger plasmid through digestion between the enzymatic sites BamHI and SpeI. The bottom panel shows the restriction sites within the multiple cloning sites indicated as a blue square in the top panel.



Appendix 3: ***The map of the pTub plasmid used to amplify the eGFP fragment.***



Appendix 4: ***The genetic map of the pBluescript KS+.***